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A search for miRNAs that regulates the expression of the atypical kinases ERK3, ERK4 and MK5

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Summary

In 2010, there were 2839 women and 13 men diagnosed with breast cancer in Norway, which was equivalent to an increase of almost 50% since 1956. The risk of developing breast cancer increases with age, and will affect one in ten women during their lifetime. Despite a substantial increase in the number of early-stage breast cancers detected due to screening-programs, there is still a need for new and improved prognostic tools and therapies for a disease with such distinctive gene expression signature and cellular composition, essential for both the biological and clinical features of the disease.

The deregulation of single-stranded RNA molecules called microRNAs (19-23 nt long) is associated to development and progression of several cancers, including in breast cancer. This has opened up the prospect of a potential role both as biomarkers, markers of cancer progression and prognosis and therapeutic targets. miRNAs regulate the cell's gene activity and protein synthesis, and their function can be both cancer-repressing and cancer-stimulating. It has been shown that miRNAs regulates the expression of the protein-coding components of many cell signaling pathways, however the complete picture of how these key cell signaling systems are regulated by miRNAs and regulate miRNA biogenesis remains to be described.

The extracellular signal-regulated kinase 3 (ERK 3) and -kinase 4 (ERK 4) are members of the atypical subgroup of MAP kinases. There is little knowledge about the upstream regulation of ERK3/4, and also their downstream targets. To this day, MAPK-activated protein kinase 5 (MK5) is the only known substrate of ERK3/4. It is believed that ERK3 is involved in cell differentiation and regulation of the cell cycle. The expression of ERK3 is up-regulated in various cancers, and ERK3 may play an important role in migration and invasive growth of cancer cells. However, the precise molecular mechanism of ERK3, ERK4 and MK5 in cancer and cancer-related signaling pathways remains to be unraveled.

In this thesis we aimed to see if we could detect a microRNA that targets and regulates ERK3, ERK 4 or MK5, either post-transcriptionally or at the translational level. By comparing the results from different target expression-experiments we sought to find correlations between the expressions of ERK3/4 or MK5 mRNA or protein and the pertaining expression of putative miRNAs with potential binding sites in the 3'UTR of these atypical kinases. Our experiments revealed the presence of ERK3 and MK5 protein in all breast cancer cell lines

tested and a possible miRNA mediated regulation of ERK3 and MK5 expression at the translational level.

Abbreviations

Innholdsfortegnelse

1 Introduction

1.1 Breast cancer

Breast cancer is a type of cancer originating from breast tissue and is regarded as a collection of breast diseases with different histopathology, genetics and genomic variations associated with various clinical outcomes (1). Different factors play a part in developing breast cancer such as lifestyle (diet, exercise and alcohol consumption), and about 5% of the cases are hereditary (2). The risk of developing breast cancer increases with age and about 80% of the diagnosed cases are over the age of 50 (3). Breast cancer affects mostly women and in 2010, 2839 women and 13 men were diagnosed with breast cancer in Norway, an increase of almost 50% since 1956. The increase in breast cancer diagnoses is partly caused by earlier detection in women participating in the organized screening program, recommended by the Norwegian health government and the WHO (2, 4). This program is voluntary for women between the ages of 50-69 years old, and is meant to detect breast cancer at an earlier stage and by such probably increase the overall survival. Today, about 90% of the diagnosed women are still alive after 5 years. The Screening program started in 1996, and went nationwide in 2005. The aim is to reduce mortality of breast cancer by early detection. However, several studies have claimed that despite a substantial increase in the number of early-stage breast cancers detected, screening is having, at best, only a small effect on the rate of death from breast cancer (5, 6).

1.2 Classification of breast cancer

Every tumor has a distinctive gene expression signature, and its cellular composition is essential for both the biological and clinical features of the disease (7). Different clinical and pathological factors are used to categorize patients with breast cancer in order to assess prognosis and determine the appropriate therapy. These include patient age, axillary lymph node status, tumor size, histological grade and lymphovascular invasion, hormone receptor status, and HER2 status (8). Breast cancer is divided into different stages to determine the course of treatment. TNM-classification describes primary tumor (T), clinical lymph node status (N) and histological lymph node status (pN), and metastasis (M) (9). Tumors are

classified by gene expression patterns, and there are five common subtypes of ductal carcinoma. Mainly, the distinction between these subtypes is tumors described as estrogen receptor (ER)-positive, called luminal A and luminal B, and tumors that are ER-negative, called basal-like, ERBB2+ and normal breast tissue-like subtype (1, 7, 10, 11).

Basal (and/or myoepithelial) cells and luminal epithelial cells are two distinct types of epithelial cells found in the human mammary gland (11). The luminal subtypes make up the hormone receptor– expressing breast cancers, and around 60-70% of invasive breast cancers are classified as "luminal group" (8). They have expression patterns resembling the luminal epithelial component of the breast, which include expression of luminal cytokeratins 8/18, ER and genes associated with active ER pathway such as LIV1 and CCND1 (also known as cyclin D1) (12). Expression of ER is essential for proliferation of luminal subtypes, and both ER and the transcription factors FOXA1 and GATA3 are crucial for the lineage-specific differentiation path in breast epithelial cells. The epithelial to mesenchymal transition (EMT) is inhibited by FOXA1, and so is cell growth. Expression of this transcription factor is associated with luminal A subtype breast cancers, and also correlates with ER expression and good prognosis (13, 14). Depending on the level of expression of other genes belonging to the proliferation cluster and HER-2/neu, the luminal-group is further divided into luminal A and luminal B (15). Luminal B tends to overexpress HER2, and about 30% are HER2-positive (whereas the luminal A group is HER2-negative). The two subtypes are also distinguished by assessing the proliferative rate (determined by Ki67 expression) and the histological grade, both of which are higher in the luminal B group (8).

The basal-like subtype is responsible for about 15-20% of invasive breast cancers. It is associated with aggressive behavior and poor prognosis and about 70-80% are triple-negative phenotypes (typically does not express hormone receptors or HER-2). Basal-like breast tumors consistently express genes usually expressed in normal basal/myoepithelial cells of the breast (16-18), including basal cytokeratins 5/6, 14 and 17, p-cadherin and caveolin 1 (10, 19-21). Many of the gene products, all hallmarks of cancer, are implicated in cellular proliferation, suppression of apoptosis, cell migration and/or invasion. Basal-like tumors also under-express genes characteristic of luminal epithelial cells of the normal mammary gland, and also genes located in the HER2 amplicon on 17q21 (8, 15). Aggressive features such as TP53 mutations and being grade III are more likely in this subtype of breast cancer than for the luminal A group (12).

Both the morphological features and the gene expression profile of basal-like breast cancers are remarkably similar to those of tumors arising in breast cancer 1, early onset gene (*BRCA1)* mutation carriers (22-26). *BRCA1* tumors are usually characterized as high-grade, highly proliferating, estrogen receptor-negative and HER2-negative. They generally express basal markers such as basal keratins, P-cadherin and epidermal growth factor receptor (EGFR), and often carry p53 mutations (27-30). BRCA1, which functions in cell cycle checkpoint responses and plays an important role in DNA double-strand break repair, contributing to the maintenance of DNA stability, may therefore have an impaired pathway in many basal-like breast cancers (12, 31).

HER-2-overexpressing tumors represent about 10-15% of invasive breast cancers (8), and express high levels of genes located in the ERBB2 amplicon on 17q21. Gene products include HER-2 and growth factor receptor-bound protein 7 (GRB7), the transcription factor GATA4, and a high level of nuclear factor (NF)-κB activation. These tumors lack expression of ER/PR and GATA3 (15). As with basal-like tumors, the HER2-overexpressed tumor's poor prognosis seems to be derived from a higher risk of early relapse among those without complete eradication of tumor cells (10, 12). As with basal-like tumors, the HER2-overexpressed tumors are more likely to have a high proportion of TP53 mutations, significantly more likely to be grade 3 and involve axillary lymph nodes than luminal A tumors (8, 12).

The normal breast tissue-like group is still poorly categorized (32), but resemble normal breast tissue samples and show high expression of many genes expressed by adipose tissue and other non-epithelial cell types, and low expression of genes characteristic of luminal epithelial genes (7, 10, 11, 15). It has been suggested that this subgroup may be a mere artifact of disproportionally high content of normal tissue contamination.

In addition to the luminal-, basal- and HER2-subtypes, at least three other molecular groups of ER-negative cancers have been described. The 'molecular apocrine' group of tumors has been claimed by some to be similar to the HER2-positive/ER-negative subtype and appears to have activation of the androgen receptor signaling. The 'interferon' subtype is characterized by high expression of interferon regulated-genes, including STAT1. And finally, the 'claudin-low' subgroup comprises tumors that are characterized by a low (or absent) expression of luminal differentiation markers, high expression of epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features (33). Clinical and

biological significance of tumors pertaining to these newly described classes remains to be determined (32).

1.3 Breast cancer cell lines

There is a necessity for the use of different model systems in breast cancer research to investigate the complex pathobiology of breast cancer, and to screen for and characterize new therapeutics. Breast cancer cell lines are widely employed, and have contributed a considerable part of our knowledge on breast carcinomas, such as insight into the deregulation of proliferation, apoptosis and migration during progression of breast cancer (1, 34). In applying breast cancer cell lines under well-defined experimental conditions, the results will generally be reproducible and quantifiable. Other advantages such as the cells being amenable to genetic manipulation and easily propagated are some of the reasons why such a model system is the principal in breast cancer research (1). Studies using rodent cells have also provided current understandings of how cell transformation occurs in human tissues. Recent evidence show that human epithelial cells require more genetic alterations to transform than their murine counterparts, suggesting that human cells in research are more relevant to human disease (35, 36).

Through gene expression profiles, breast cancer cell lines can be divided into three groups; luminal, basal-A and basal-B. The luminal subtype comprises ER-positive cell lines and is characterized by the expression of ERα-regulated genes, as well as genes associated with luminal epithelial differentiation like GATA3 and FOXA1 (37). Cell lines in the basal-A subgroup are ER-negative, express basal epithelial gene markers and some luminal epithelial markers, as well as a marker of cancer stem cells (PROM1) (37, 38). The basal-A subtype is also associated with the breast cancer 1, early onset gene signatures (BRCA1), and the erythroblast transformation specific pathway (ETS-pathway) (37). Similar to the basal-A subgroup, the basal-B subgroup consist of ER-negative cell lines. The basal-B subgroup generally express basal epithelial gene markers (like Moesin, ETS1, CAV1 and EGFR) (37, 39, 40), and show features of aggressive tumors (plasminogen activator and TGFB1) (41, 42), invasiveness (epithelial mesenchymal transition) (43), and markers for normal breast and breast cancer stem cells (44). A subset of this group also lacks the expression of the basal cytokeratin markers KRT5 and KRT17 (37).

Tumor subtypes show close similarities to breast cancer cells when comparing them to the tumor's gene expression patterns. Where the luminal-A or luminal-B tumors resemble the luminal cell lines, most basal-A cell lines resemble basal-like tumors, and most basal-B cell lines resemble either basal-like or ERBB2 tumors (31). A reverse comparison of breast tumors to the cell line subtypes show that the ERBB2 subtype tumors show conformity to either luminal or basal-A cell lines (37). The fact that the cell lines mirror many, but not all, of the genomic and biological properties of primary tumors, may be due to the absence of normal epithelial or stromal cells affecting the genetic profile of breast cancer cell lines. Lack of stromal or physiological interactions or signaling in cell culture may also account for some of the differences. Culture conditions selecting for subpopulations of breast tumors may be a cause for the differences between genome aberration patterns for the basal-like and luminal clusters in the cell lines, and these subtypes in primary tumors. Hence cell cultures show a smaller variation in gene expression profiles than breast tumor subtypes. Yet, cell lines still largely reflect the genomic heterogeneity and the recurrent genome copy number abnormalities found in primary tumors (45).

1.4 Protein kinases and phosphatases

Protein kinases and phosphatases play important roles in regulating and coordinating aspects of metabolism, gene expression, cell growth, motility, differentiation and division in normal cells (46). The protein kinases are important enzymes in cellular signaling, and carry out their action by covalently attaching phosphate to side chains of the amino acids tyrosine, serine and threonine of specific proteins. When enzymatic proteins are phosphorylated, this will control their activity and interactions with other molecules, their location in the cell and also their susceptibility for degradation proteases. These are proteins that remove the previously transferred phosphates from the protein-substrates (47).

An extracellular signal can be transduced and amplified in cells through the protein kinase cascades. Receptors activate tyrosine phosphorylations, and the protein kinases tightly regulate the intracellular signaling pathways (48). A deregulation of these processes by the occurrence of mutant alleles disrupting the signaling might lead to malignant changes in cell differentiation, division, motility and apoptosis. The protein kinases can be subdivided into

three categories based upon their catalytic specificity: those specific for tyrosine, those specific for serine/threonine and those specific for both tyrosine and serine/threonine (49, 50).

1.5 Protein tyrosine kinases

There are two classes of protein tyrosine kinases (PTKs) present in cells: the transmembrane receptor PTKs and the non-receptor PTKs. The non-receptor PTKs (NRTKs) are cytoplasmic proteins triggered by the receptor tyrosine kinases (RTKs) (51). Receptor protein tyrosine kinases are transmembrane glycoproteins that after activation by ligand-bidning, induces dimerization and activation of the receptor tyrosine kinases, which autophosphorylate and transfers the signals to the cytoplasm (48).

Cytoplasmic signaling pathways that are activated by PTKs include the Ras/Raf mitogen-activated protein kinase pathway, the phosphoinositol 3-kinase/Akt pathway, the signal transducer and activator of transcription 3- pathway and the protein kinase C -pathway. Signals are transferred from membrane receptors into the nucleus by intracellular mediators in these pathways, where they culminate and cause altered DNA synthesis and cell division as well as effects on different biological processes such as cell growth, migration, differentiation, and apoptosis (50).

The development of cancer may happen through several mechanisms in cells. Many factors contribute to the activation of cell cycle and other processes involved in cancer, such as mutations in the tumor suppressor p53, overexpression of different growth factors (like VEGF or TGF-α) or growth factor receptors (like PDGFR or EGFR), or the deregulation of kinases (like ABL) and activation of oncogenes (like the Ras/Raf pathway) (52).

1.6 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) convert extracellular stimuli into a wide range of cellular responses. They are protein Serine/Threonine kinases, and MAPK pathways regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation (53).

The extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAPK-isoforms, c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3) and ERK5 all belong to the conventional MAPKs (54, 55). Atypical MAPKs comprise ERK3/4, ERK7, and Nemo-like kinases (NLK), and do not share many characteristics of conventional MAPKs (56, 57).

The MAP-kinase cascade consists of sequentially acting kinases: a MAPK, a MAPK kinase (MAP2K), and a MAPKK kinase (MAP3K) (see Figure 1). Activation of the MAP3Ks is often through phosphorylation or in response to extracellular stimuli through interaction with a small GTP-binding protein of the Ras/Rho family. The activation of MAP3K leads to a MAP2K being phosphorylated and activated, which then in turn phosphorylates the tyrosine and threonine residues on a MAPK, activating it. Phosphorylation of these Thr/Tyr-residues is essential for enzymatic activities(58) .

MAPKs can phosphorylate several substrates, including the protein kinases called MAPK activated protein kinases (MAPKAPKs or MKs), which comprises of the p90 ribosomal S6 kinases (RSKs), mitogen- and stress- activated kinases (MSKs), MAPK-interacting kinases (MNKs), MAPK-activated protein kinase 2/3 (MK2/3) and MK5. These kinases represent additional enzymatic amplification steps in the MAPK catalytic cascades (47, 56).

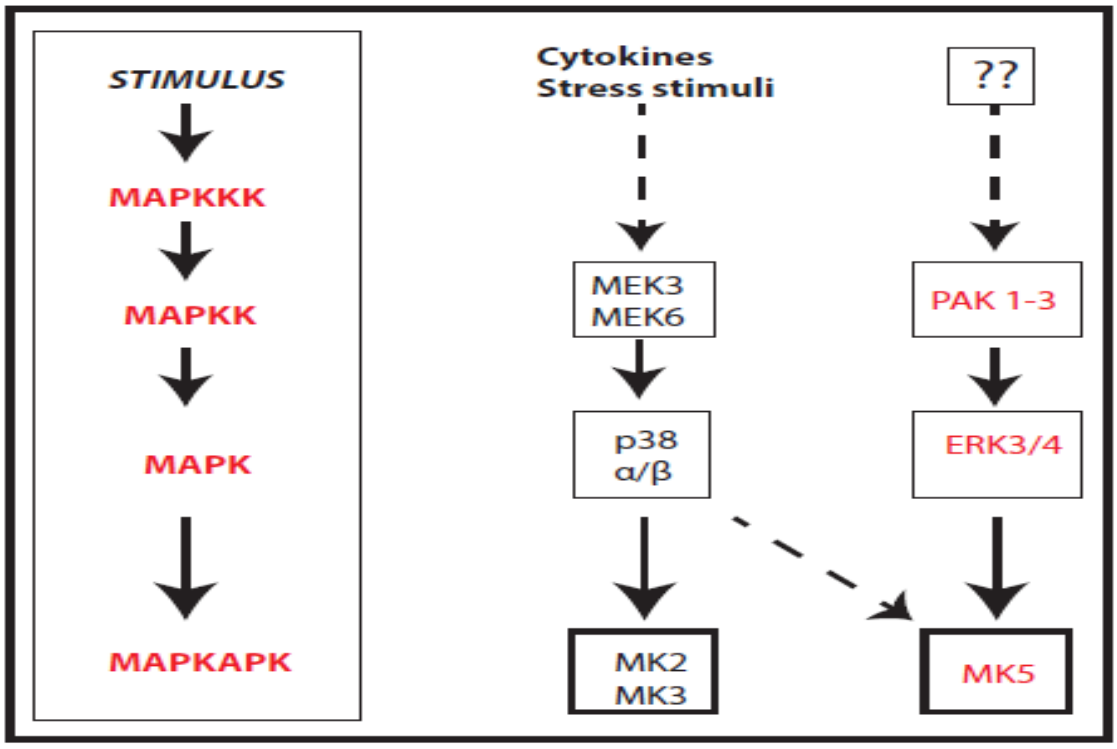

Figure 1. MAPK signaling cascade leads to activation of the MAPKAPKs. This figure is showing a part of the different MAPK pathways, which is activated through stimulation from various cellular stress signals, mitogens and cytokines. Activation of the pathway leads to phosphorylation and activation of subgroups in the cascade, including the MAPK-activated kinase, MK5. The dotted line represents the fact that substrate regulation of MK5 by p38 α/β is not yet fully demonstrated. This figure is modified from Cargnello M. and Roux P. 2011 (56) and Déléris et al. 2010 (59).

1.7 Atypical MAP kinases

Atypical MAP kinases are divided into ERK3 (MAPK6), ERK4 (MAPK4), nemo-like kinase (NLK) and ERK7 (MAPK15). One distinctive feature that sets the conventional MAP kinases apart from the atypical MAP kinases ERK3 and ERK 4 is that tyrosine in the activation loop (Thr-Xaa-Tyr), which is the site of activating phosphorylation by the MAP2K family members, is replaced with a glutamic acid residue. The activation loop in ERK3and ERK4 therefore contains a single phospho-acceptor site (Ser–Glu–Gly, or SEG). Subdomain VIII of the kinase domain also contains an altered sequence in ERK3 and ERK4. As the only known kinases in the human genome, that domain contains the sequence Ser–Pro–Arg instead of Ala–Pro–Glu (57).

ERK3 is found to be a highly unstable protein, degraded by the proteasome-enzyme, and is found in both the cytoplasmic and nuclear compartments in exponentially proliferating cells. Unlike ERK3, ERK4 is a stable protein and is predominantly localized in the cytoplasm. They have both been implicated in cell regulation and cancer metastasis and migration (60, 61). Binding of ERK3/4 to MK5 is accompanied by phosphorylation and activation of this MAPK-activated protein kinase (57, 62). ERK3 has also been seen as a physiological target for MK5 (63).

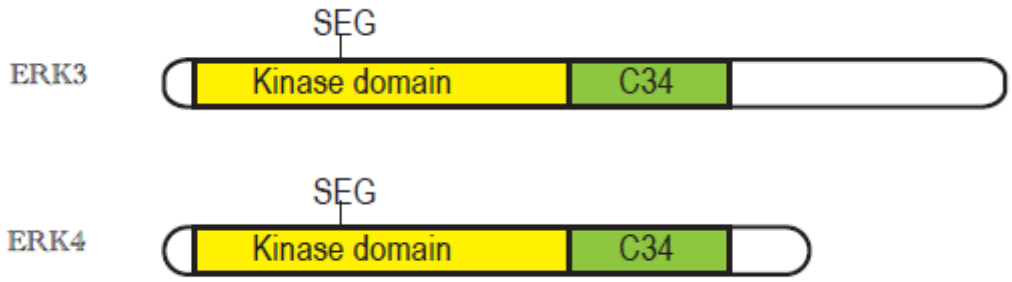

Figure 2. Schematic representation of the structure of the atypical MAP kinases ERK3 and ERK4. MAP kinases consists of a kinase domain (in yellow) flanked with an N- terminal, and a C-terminal with different lengths. C34 is a conserved region in ERK3/4 (56, 57).

MK5, a 54 kD protein part of the MAPK-activated protein kinases (the MAPKAPKs or MKs) is activated through phosphorylation of its threonine 182 (T182) by JNK, ERK2 and p38 MAPKs (64). In cells, MK5 is found both in the nucleus and the cytoplasm, but mainly in the nucleus of resting cells (65). With MK5 being a part of the atypical MAPK cascade, the structure contains a region for binding ERK3 and ERK4 in the C-terminal. This is an extension from the NLS of a 100 amino acids, which differs from the conventional MAPKAPKs MK2 and MK3. The catalytic domain of MK5 is found in the N-terminal domain (see Figure 3) (66, 67). There is also a docking motif for the p38 MAPK, and a p38-isoform may activate MK5 *in vivo (68).* The nuclear export sequence (NES) and the nuclear localization sequence (NLS) resides within the C-terminal (65).

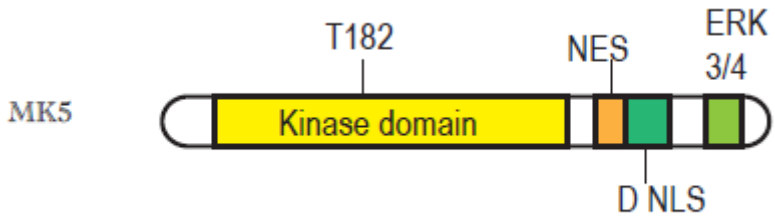

Figure 3. Schematic representation of the structure of the MAPK-activated protein kinase MK5. NES: nuclear export signal, NLS: nuclear localization signal, D: D domain/MAPK docking site, ERK3/4: docking site for ERK3/4 (56, 69).

1.8 Non-protein coding RNAs

For many years it was generally assumed that most genetic information was transacted by proteins, however, it is now clear that the majority of the genomes of mammals and other complex organisms is in fact transcribed into non-protein coding RNAs (ncRNAs). Non-protein coding RNAs do not encode proteins, but function directly at the level of the RNA in cells, and the importance of this diverse class of molecules is widely recognized (70-73). Although only 2% of the mammalian genome encodes mRNAs, the vast majority is transcribed, largely as long and short ncRNAs (74-76). Their functions include DNA replication, chromosome maintenance, regulation of transcription, regulation of translation, RNA processing (including RNA cleavage, -re-ligation, -modification and –editing), regulation of mRNA stability and regulation of stability and translocation of proteins (73, 77-81).

Non-protein coding RNAs include both “housekeeping” RNAs, such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), as well as regulatory ncRNAs. Regulatory ncRNAs are generally grouped into two major classes based on transcript size; long ncRNAs (lncRNAs) and small ncRNAs (82-84).

1.9 Small regulatory RNAs

At least three classes of small RNAs are encoded in our genome, based on their biogenesis mechanism and the type of Ago protein that they are associated with: microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs or esiRNAs) and Piwi-interacting RNAs (piRNAs).

miRNA is by far the most studied and best understood among the three classes. miRNAs are endogenous, evolutionary conserved, single-stranded RNA molecules (18–25 nt) that are involved in specific regulation of gene expression in eukaryotes. The first miRNA (called lin-4) was discovered by Ambros and coworkers in 1993, from *Caenorhabditis elegans*, however it took seven years before the second miRNA was discovered (let-7). This observation triggered the construction and characterization of several large-scale cDNA libraries enriched for small RNAs and led to the identification of thousands of miRNAs in organisms as diverse as viruses, plants, worms, and mammals (85-89). The number of miRNAs in the human genome is currently estimated to be 1600 precursor- and 2042 mature miRNAs (90).

miRNAs are generated from local hairpin structures by two RNase III proteins; Drosha and Dicer. This generates mature miRNAs of ~22 nt that are subsequently bound by Ago-subfamily proteins. miRNAs generally target mRNAs and function as post-transcriptional regulators. It is predicted that the miRNA genes comprise 1–2% of the human genome and that miRNAs control the activity of about 50% of all protein-coding genes (91, 92).

siRNAs differ from miRNAs in that they are derived from long double-stranded RNAs (dsRNAs) and are dependent only on Dicer and not on Drosha (83, 93, 94). siRNAs are also considered to be generally exogenous RNA molecules that are taken up by cells, or enters via vectors like viruses. They are also slightly shorter (~21 nt) than miRNAs and, at least in animals, siRNA typically binds with complete complementarity to its mRNA target and regulates gene expression by inducing mRNA cleavage (95).

piRNAs are the longest of the three classes (24–31 nt in length), and are associated with Piwi-subfamily proteins. Intriguingly, the biogenesis of piRNAs does not depend on Dicer (96).

piRNAs are highly abundant in germ cells and have been implicated in transposon silencing through heterochromatin formation or RNA destabilization (97).

1.10 MicroRNA Biogenesis

The biogenesis of a miRNA starts in the cell nucleus. The pri-miRNA can be several hundreds or thousands of nucleotides (nt), and are usually transcribed by RNA polymerase II (98, 99), or in some cases by RNA polymerase III (100). The primary miRNA transcript is characterized by a stem loop structure, and is further processed in the nucleus by an RNase III endonuclease, Drosha, which cleaves both strands of the stem at sites near the base of the stem loop and releases the pre-miRNA. Drosha is part of a large protein complex called the microprocessor, containing the DiGeorge syndrome component region gene 8 protein (DGCR8). Drosha serves as the catalytic unit, while DGCR8 recognizes the pri-miRNA and stabilizes it's interaction with Drosha (98, 101). The resulting pre-miRNAs will be 60- to 100 nt long hairpin structures with a 5'phosphate and ~2-nt 3' overhang at the base (98).

After being processed in the nucleus, this pre-miRNA is recognized by Ran-GTP and the export receptor Exportin-5 transporter-complex, and actively exported into the cytoplasm. The nuclear cut by Drosha defines one end of the mature miRNA. The other end is processed in the cytoplasm by the enzyme Dicer (98). Dicer is a multidomain protein that consists of a double stranded binding domain (dsRBD), a RNAhelicase/ATPase domain, a PAZ (Piwi/Argonaute/Zwille) domain, two neighbouring RNase III-like domains (RIIIDs) and the DUF283 domain. The dsRBDs and RIIDs gives Dicer a preference for processing double stranded RNA substrates with 3'overhangs, while the PAZ domain recognizes the 3'-protruding ends of the substrates and cleaves the double stranded region two helical turns away from the stem-loop. The result is a miRNA duplex (about 22 nt long), which includes the mature miRNA guide strand and the complementary passenger strand (miR-3p/miR-5p) (98, 102).

The transactivating response RNA-binding protein (TRBP) and protein activator of protein kinase R (PACT) facilitate Dicer-mediated cleavage of the pre-miRNA by binding and stabilizing Dicer (103, 104). They are also important regulatory factors that contribute to both

substrate and cleavage specificity, and the selection of which miRNA strand that is transferred to the RNA-induced silencing complex (RISC) or miRgonaute (98, 105). Indeed the PACT-TRBP–Dicer complex provides a platform for RISC assembly and aids recruitment of Argonaute proteins (in humans, usually Ago2), the catalytic enzyme of RISC required for miRNA processing (103, 104, 106).

The Ago proteins are central for RISC function and contain two conserved RNA binding domains: a PAZ domain that bind the single stranded 3’ end of the mature miRNA and a PIWI domain that interact with the 5’ end of the guide strand. These domains bind the mature miRNA and position it for interaction with a target mRNA. Some Ago proteins (for example Ago2), cleave target transcripts directly while others recruit additional proteins to achieve translational repression (107). RISC with incorporated miRNA is generally referred to as "miRISC”, and this complex can now, dependent on the sequence complementarity between the miRNA and its mRNA target, regulate the expression of target mRNAs. Binding to target mRNAs usually happens through partial complementarity and lead to a coupled mRNA degradation and inhibition of translation (108, 109).

Mirtrons are relatively recently characterized cellular RNA interference (RNAi) effectors produced via a non-classical miRNA pathway. They are generated by using a splicing and intron lariat-debranching enzyme machinery that bypass Drosha cleavage. The dependence on the remainder of the RNAi pathway is variable, however the mirtron pathway generally converges with the miRNA pathway during export of hairpin structures from the nucleus to the cytoplasm by Exportin-5, and processing by Dicer (110, 111).

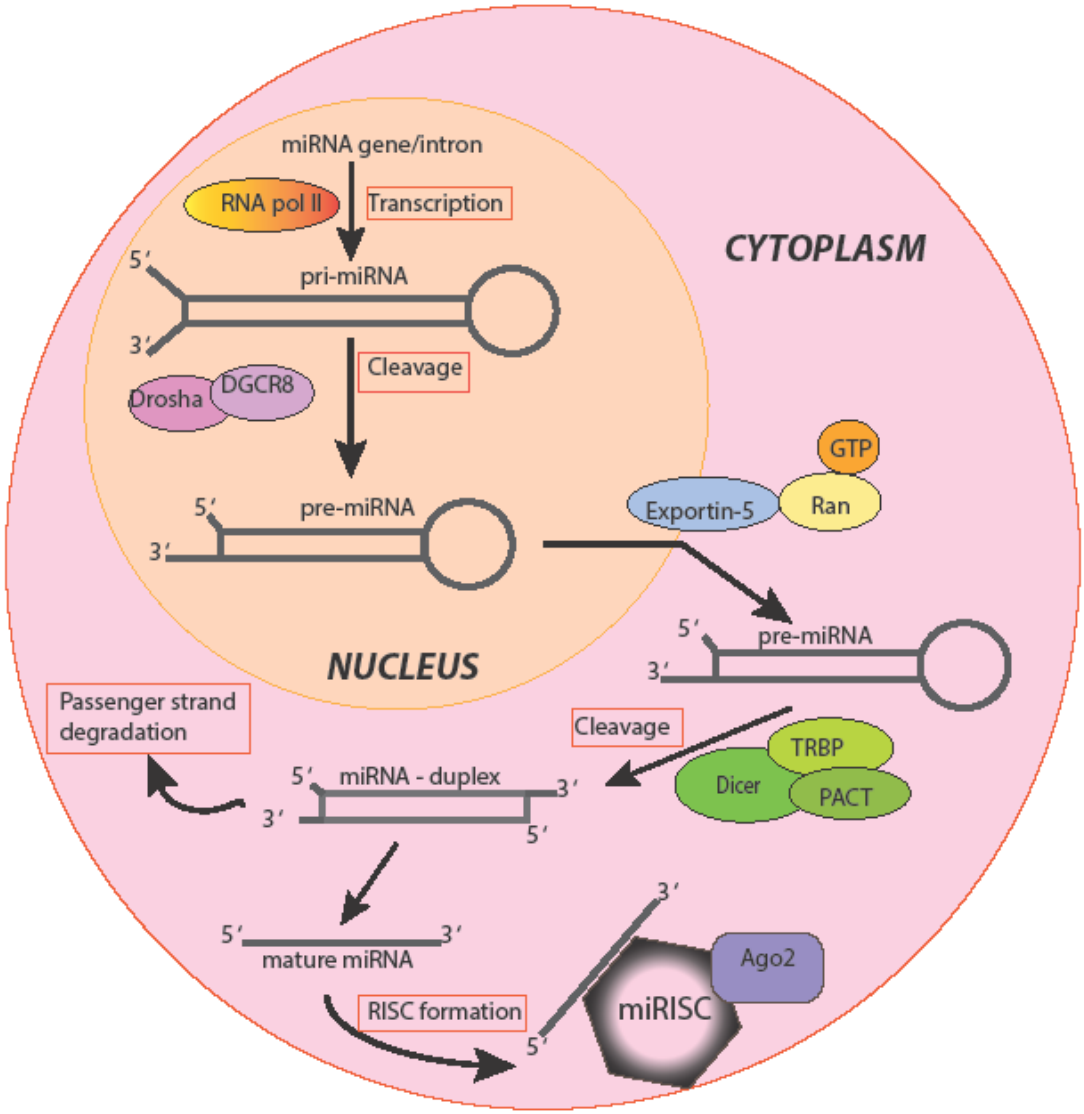

Figure 4. The miRNA biogenesis pathway. This recognized maturation pathway of mammalian miRNAs starts in the nucleus with RNA polymerase II cleaving the primary transcript (pri-miRNA), which is further processed by the microprocessor complex Drosha-DGCR8. This generates the pre-miRNA which is exported to the cytoplasm by Exportin-5-Ran-GTP. The RNase Dicer-complex cleaves the miRNA hairpin structure into its mature length of 19-25 nt. The duplex is unwound, and the guide strand is incorporated into RISC while the passenger strand usually is degraded. The miRISC complex can then be guided to its target, and achieve gene silencing (112, 113).

1.11 MicroRNA genes

miRNAs are present in all plants and animals that have been studied so far, suggesting an early origin of these RNA-molecules (78). Many miRNAs may be clade- or organism-specific, though the number of miRNAs in a genome is still subject for discussion (114). Although the number of of miRNAs in the human genome is still in some flux, it is currently estimated to be 1872 precursor- and 2579 mature miRNAs (115)

Approximately 30% of miRNA genes can be found in intergenic regions (between genes), distant from earlier annotated genes (78). These miRNAs have their own promoter region (116, 117), and their expression is thought to be regulated by the same molecular mechanisms that control the expression of protein-coding genes.

The majority of miRNA genes are located in defined transcription units, predominantly located inside introns and usually oriented on the same DNA strand of the host gene (118). This involves both protein-coding genes and non-protein coding genes. Most intragenic (inside genes) miRNA genes are thought not to be transcribed by their own promoters, but to have the same orientation and promoters as their host genes. This might imply that at least for the protein-coding genes, they serve as hosts for co-transcribed miRNAs, giving a coordinated expression of miRNAs and proteins and a potential co-regulation of host mRNA splicing and miRNA biogenesis (78, 119).

Exon-derived miRNAs are quite rare and are generally encoded by exons of long non-protein-coding transcripts, also called mRNA-like noncoding RNAs (mlncRNAs) (120).

Introns that comprise the exact sequence of the pre-miRNA with splice sites on either side are called mirtrons. These are spliced out of the host transcripts and form secondary structures resembling those of pre-miRNAs. In this manner the debranched introns enter the miRNA-processing pathway without Drosha-mediated cleavage (121).

Approximately 50% of mammalian miRNA loci are found in close proximity to other miRNAs. MicroRNAs that are clustered close together in the same genome may be expressed from one primary transcript. Such polycistronic transcripts are several kilobases long in mammals, and represent over 40% of human miRNAs (114). MicroRNAs within a cluster are often related to each other (120), and have highly similar expression profiles. One possibility

for this is that the transcription is driven by a single promoter. Yet another possibility might be that each miRNA is transcribed by their own promoter from the ~70 nt precursor transcript, but that the transcription is controlled by a common enhancer (122). Most mammalian miRNA genes have multiple isoforms and this is probably caused by gene duplications. The largest families of human miRNA isoforms include let-7 (14 members) and miR-30 (6 members). miRNA isoforms are divided in two types: (1) When the mature miRNAs are of nearly identical sequences (usually differing by 1–3 nt), the families are designated with a letter (e.g. let-7b and let-7c). (2) When miRNA genes produce identical mature miRNA from different precursor genes, the families are designated with a number implying that each gene produces the identical mature miRNA (e.g. let-7a-1 and let-7a-2). In the latter case each isoform is usually located on different chromosomes. The isoforms (isomirs) often have identical sequences at nucleotide positions 2–7 (called the seed region) relative to the 5' end of the miRNA. Because these nucleotides are critical in base pairing with the target mRNA, the isomirs are thought to act redundantly. Still, the 3' sequences of miRNAs also contribute to target binding and because the expression patterns of these sister miRNAs are often different from each other, members of the same seed family might have distinct roles in vivo (123).

1.12 The mechanism of miRNA mediated gene silencing

There is an ongoing search for the exact mechanism by which miRISC regulates translation of its target genes (95, 124-126). In animals the theory was originally that miRNAs repressed translation, but had no influence on the level of their mRNA targets. In plants on the other hand, miRNAs were thought to promote mRNA cleavage and degradation because of their perfect miRNA-mRNA complementarity, excluding translational repression which is thought to stem from mismatches and bulges. However, it has now become more clear that also miRNA-mediated mRNA degradation occurs in animals, initiated by deadenylation and decapping, followed by exonucleolytic digestion of the mRNA (95, 127). Translational repression has further been proposed to occur in four different ways: repression at the translation initiation step (128-131), cotranslational peptide degradation (132), increased premature termination (ribosomal drop-off) (133) and impaired elongation (125). In any case

it has become clear that miRNAs cause mRNA degradation or translational repression in both plants and animals, but there are still insecurities as to whether silencing occurs with translational repression at the initiation step or with degradation of mRNA targets at following stages (95).

A paper published in 2010 by Bartel and colleagues concluded that the main reason for reduction in protein expression seems to be miRNA-mediated destabilization of mRNA targets. By using ribosome profiling to compare the protein production and mRNA levels, they discovered that around 84% of the lowered amount of protein was due destabilization of target mRNAs. Furthermore, the fraction of mRNA not degraded was also translated less efficiently (134).

In the first step of miRNA mediated gene silencing process, the RNA-induced silencing complex (RISC) recruits one strand from the miRNA/miRNA* duplex by loading it onto an Ago protein at the core of the miRISC (135). This miRNA-strand, termed the guide-strand, can now recognize the sequence of its RNA target (136). The mechanism for gene regulation by miRISC seems to be influenced by the degree of complementarity between the guide-strand and its target mRNA (95). Several computational and biochemical studies indicate that the specificity of target recognition by miRNAs are dependent on nucleotides 2 – 8 of the guide strand, also known as the 'seed' region. At the 5' end of the guide strand, nucleotides 2 – 6 are exposed so that the seed region can interact (by Watson-Crick base pairing) with the target mRNA and provide slicer cleavage at a fixed distance due to perfect complementarity in the cleavage site (135). Benjamin P. Lewis and coworkers published a paper in 2003 which describes an algorithm called TargetScan, for predicting vertebrate miRNA targets. Their analysis reinforces the thought that the seed region is the most conserved portion among related miRNAs, and has the best inclination for matching numerous conserved segments in untranslated regions of mRNA targets (137). Yet, there are examples of functioning miRNAs that lack high binding degree and complementarity in the seed sequence, though this is normally required for repression (138). Other regions such as the highly conserved 3' complementary site or "centered sites" facilitate such action (139). This makes it possible for one miRNA to target several mRNAs, as well as different miRNAs regulating the same protein-coding gene (140).

The actions of miRNAs are mediated by argonaute-proteins (AGOs). After binding of the miRISC to an RNA-target, regardless of the mode of translational repression, the AGOs will

co-localize to cytoplasmic foci known as processing bodies (P-bodies) (141-143). P-bodies entail different enzymes that contribute to post-transcriptional decapping and degradation of translationally silenced mRNAs in the 5' –to 3' direction (142, 144). It seems that either as a cause or a consequence of inhibited protein synthesis, repression of translation will cause an association of mRNAs to P-bodies (141).

The first and rate-limiting step of miRNA-mediated mRNA degradation is poly(A) removal by the deadenylase CCR4-NOT, which is accelerated by RISC (145). miRNA-mediated repression is also dependent on the GW182 proteins interacting with AGOs, and recruiting additional effector complexes required for silencing in human cells (136, 146). GW182 is a protein that comprises multiple glycine(G) - tryptophan(W) repeats, an RNA recognition motif (RRM), and functions in RISC-mediated silencing correlating to maintenance of P-bodies (147, 148). The silencing domain of GW182 proteins interact with the poly(A) –binding protein (PABP) and the deadenylase complexes. Recruitment of the deadenylase complex releases PABP from the mRNA poly(A) tail, causing a halt in mRNA circularitzation and enabling translational repression, deadenylation and subsequent mRNA degradation (149, 150).

Athough some studies indicate that miRNAs repress translation before deadenylation and mRNA decay, many uncertainties still surround the subject (151). Translational repression and deadenylation may be either independent or co-dependent mechanisms for repression, however both require the interactions between the GW182 proteins and deadenylases (150).

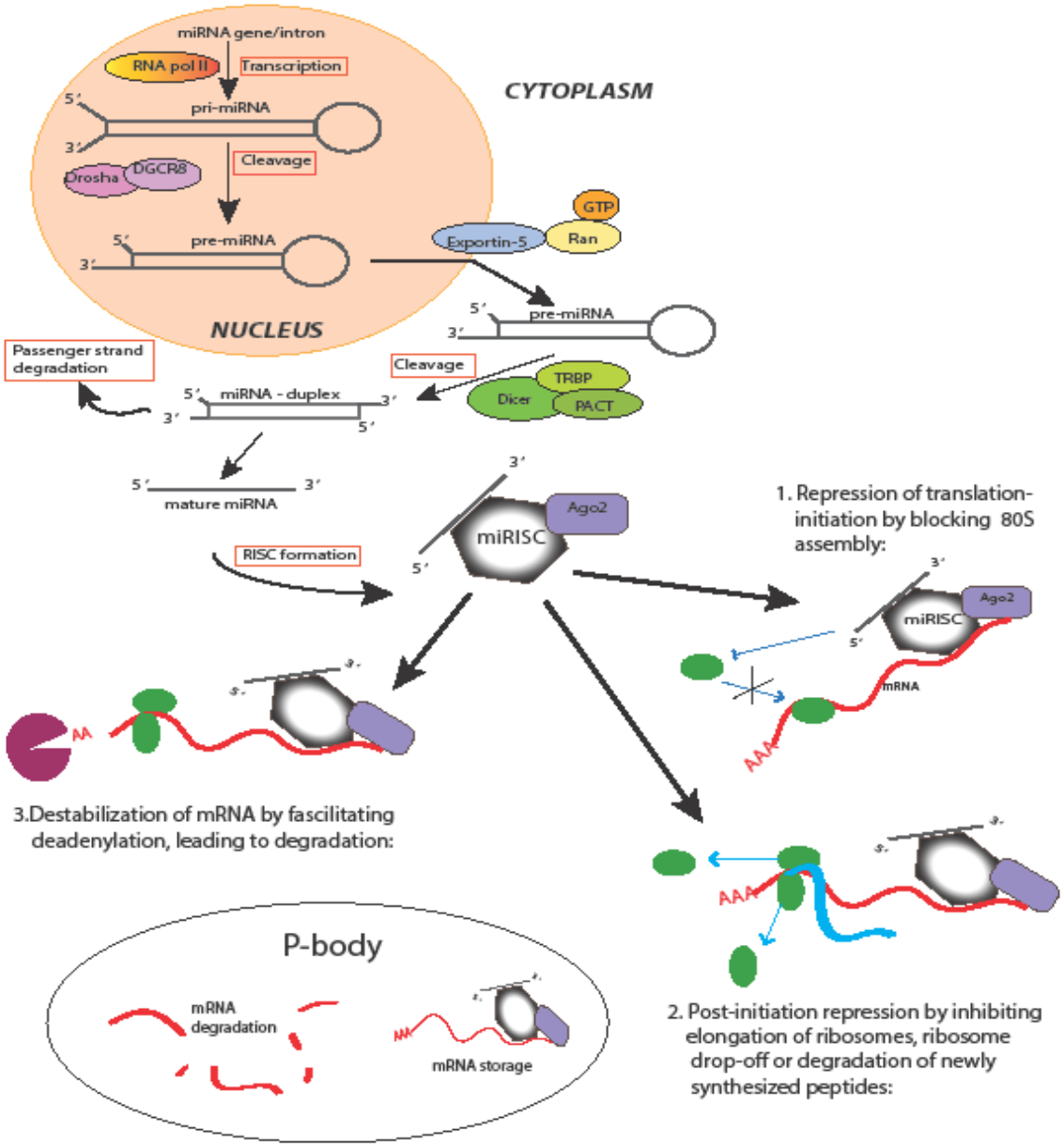

Figure 5. miRNA mediated gene silencing. The miRNA guides RISC to its target mRNA. miRISC binds to the target 3'UTR and inhibits gene expression. The direct effect on translation occurs through either 1) repression of translation-initiation, resulting in prevention of ribosome association, or 2) inhibition of translation post-initiation, including premature ribosome drop-off, stalled elongation and protein degradation. Indirect effects on translation include 3) promoting deadenylation, resulting in potential degradation of the mRNA. P-bodies are cytoplasmic foci enriched with mRNA-dagradation factors, where mRNAs might be stored for subsequent use, or deadenylated and degraded (152, 153).

1.13 MicroRNAs and breast cancer

The first miRNAs were discovered in 1993(154). However it took another seven years before it was uncovered that miRNAs are highly conserved in nature and miRNAs became recognized as a distinct class of biological regulators (155). Two years later, in 2002, the first link between miRNAs and cancer was established. Columbus University researcher Carlo Croce identified two microRNAs, miR - 15 and miR- 16, located in a gene cluster on chromosome 13 that was found to be deleted in most cases of chronic lymphocytic leukemia (CLL). The down-regulation of these microRNAs was found in around 65% of CLL-patients, caused by a germ-line mutation in their primary precursor. The assumption that miR-15 and miR-16 act as tumor suppressors was later confirmed by Cimmino, Calin and Fabbri et al. They showed that the expression of B cell lymphoma 2 (BCL2), a gene inhibiting cell death, was down-regulated post-transcriptionally by miR-15 and -16, causing activation of the intrinsic apoptosis pathway (156).

Important steps in cancer development involve initiation, promotion, malignant transformation, progression and metastasis (157). miRNAs have been shown to influence biological pathways controlling all these steps, and interaction between mRNA and miRNAs affect the fate of both normal and diseased cells (158). A study conducted by Calin and coworkers found that over 50% of miRNA genes are located in fragile genome-sites and cancer related regions, and that several of the miRNAs showing low expression levels in cancer samples were located in such deleted regions (159).

In 2005, Iorio et al. reported abnormal expression of miRNA in human breast cancer. They also identified a correlation between miRNAs and specific features of breast cancer such as proliferation index, expression of estrogen/progesterone receptors, tumor stage and angiogenesis (160).

miRNAs have been shown to regulate the expression of both oncogenes and tumor suppressor genes (161). miRNAs that negatively regulate transcripts encoding tumor suppressor genes are often calles oncomiRs. Oncosuppressor miRNAs on the other hand, have oncogenes as targets and these miRNA-genes are located in loci subjected to deletions, mutations or other abnormalities causing a reduced level of these anti-cancer miRNAs (162).

miR-21 is an example of an oncomiR that is often up-regulated in breast cancers and directly targets tumor suppressor genes like tropomysin 1 (TPM1), Phosphatase and tensin homolog (PTEN) and Programmed Cell Death 4 (PDCD4) (163-165). Furthermore miR-21 has been suggested to be involved in the regulation of the RAS oncogene and gene family, transforming growth factor beta 1 (TGF-β) protein and receptor, and the previously mentioned BCL-2, all components of regulating pathways in cell growth and differentiation, adhesion and migration, and cell survival (166-168). High expression of miR-21 is correlated with advanced tumor stage, lymph node metastasis, and poor survival in breast cancer patients, suggesting that miR-21 might serve as a molecular prognostic marker for breast cancer and disease progression (166).

Another microRNA acting as an oncomiR in breast cancer is miR-155, which is also described in lymphoma, viral infections, cardiovascular disease and in solid cancers (169). As with miR-21, miR-155 is up-regulated in breast cancer (160), and has a role in cell proliferation and apoptosis, as well as mediating TGFβ-induced EMT (169, 170). miR-155 promotes migration and invasion of mammary gland epithelial cells by targeting the anti-metastatic protein, RhoA (171), and activates inflammation-associated tumorigenesis by targeting the tumor suppressor gene SOCS1 (suppressor of cytokine signaling 1) (172). There has also been reported associations between increased miR-155 expression and metastasis, invasiveness and high tumor grade, as well as an inverse correlation between miR-155 and FOXO3A, associated with resistance to radio,- or chemotherapy (170, 173). Moreover, miR-155 has been found to be significantly up-regulated in basal-like and estrogen receptor negative (ER-) tumors (163), and to have a close association with the breast cancer susceptibility gene (BRAC1) (174). This links high miR-155 expression to both invasiveness and recurrence of breast tumors and poor prognosis, and a possible role as a prognostic marker (169).

miR-34a is a member of the miR-34 family (miR-34a, -b and -c), but is transcribed from an individual chromosomal locus. Although this region is lost in some cancer types (175), reduced or diminished expression could also be due to mutations in the tumor suppressor p53 which positively regulates the transcription of miR-34a (176). The tumor suppressor actions of miR-34a involves silencing of target-oncogenes such as cyklin dependent kinase 6 (CDK6), leading to apoptosis, cell cycle arrest and a halt in tumor cell proliferation (177, 178). In breast cancer there seems to be a correlation between high levels of miR-34a

expression and a lower risk of metastasis and disease recurrence, highlighting miR-34a as a possible predictor of a positive clinical outcome (179).

Similar to miR-34a, miR-31 also show low expression levels in aggressive human breast cancer and inhibits several steps in the metastasis cascade, such as metastatic colonization, local invasion and survival of cancer cells at distant sites. miR-31 repress several metastasis-promoting genes, and it has been demonstrated that overexpression of miR-31 alone is sufficient for inhibiting metastasis in otherwise aggressive breast cancer cells while inhibition leads to metastasis of otherwise non-aggressive breast cancer cells (180).

The let-7 family members are also a group of tumor suppressor miRNAs that is frequently down –regulated in breast cancer (181, 182). The exact role in tumorigenesis is not yet fully understood, but let-7 members appear to play a major role in regulating stemness in breast cancer (183). and are also implicated in the negative regulation of several other oncogenes involved in regulation of cell cycle, -differentiation and -apoptotic pathways (184-186).

Overall miRNA expression is generally deregulated in all human cancers, including breast cancer, and several miRNAs have been identified as possible biomarkers and/or characterized as essential regulators of breast cancer development (187). This abnormal expression will, accompanied by advancements in individual miRNA profiling technologies, most certainly facilitate a turn towards a more personalized cancer medicine in the future (157, 188).

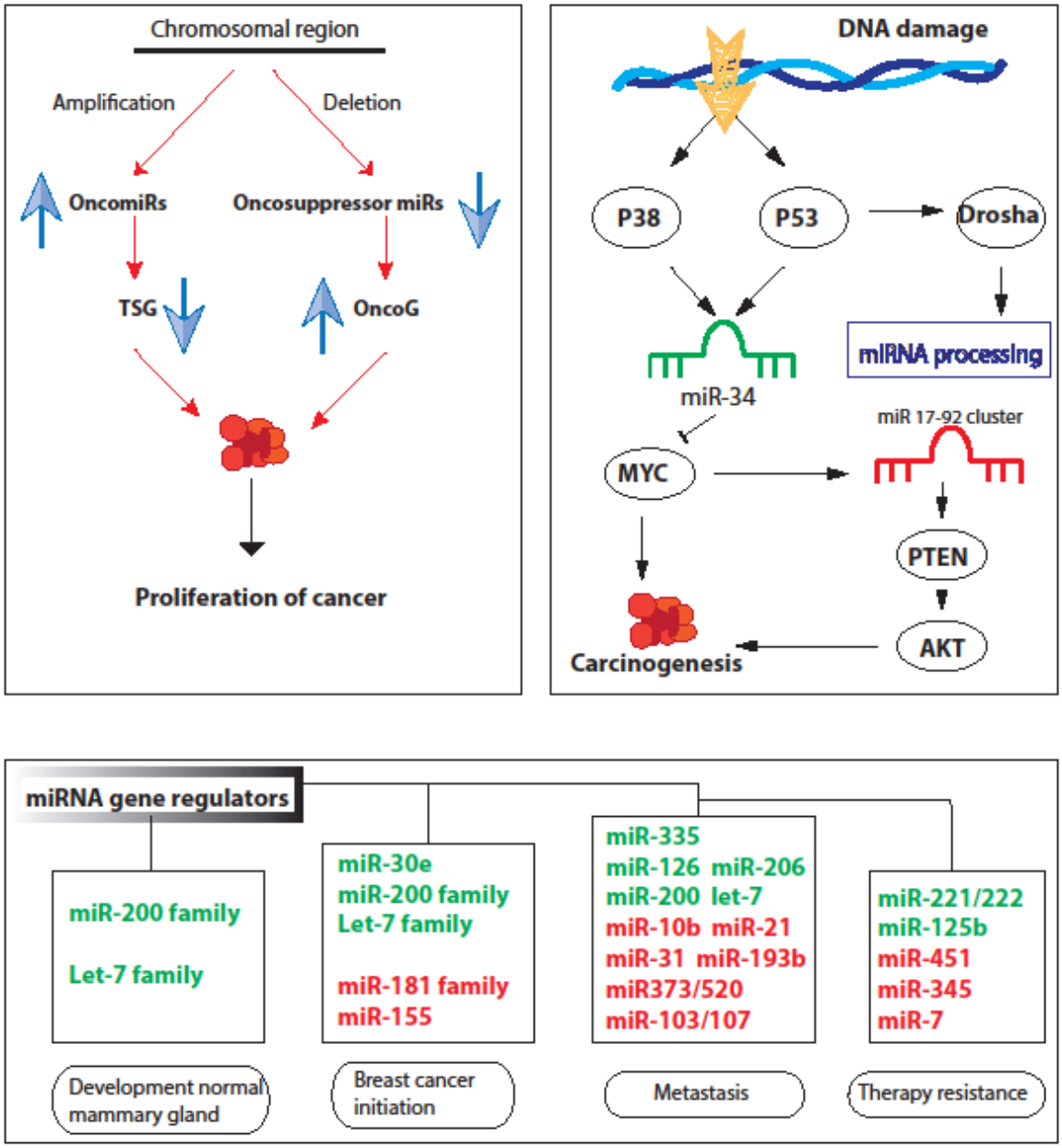

Figure 6. The role of oncosupressor miRNAs and oncomiRs in cancer proliferation, and an overview of some miRNAs reported to act as regulators in the DNA damage response, development of normal mammary gland, breast cancer initiation, metastasis and therapy resistance. TSG: tumor suppressor gene, OncoG: oncogene. Suppressor miRNAs are shown in green, and oncomiRs are shown in red. The figure is modified from (155, 158, 189).

2 Materials and methods

2.1 Cell lines

The cell-lines used in this thesis were: AU 565, DU 4475, HCC 1187, HCC 1569, HS 578T, MA11, MCF 7 and MD-AMB-231.

The AU565 cell line was derived at the Naval Biosciences Laboratory, Oakland, CA, from a pleural effusion of a 43 years old, Caucasian, female patient with breast carcinoma. She had been treated with radiation, steroids, Cytoxan and 5-fluorouracil. The AU565 cell line amplifies and overexpresses the HER-2-neu oncogene, and expresses the HER-3, HER-4 and p53 oncogenes, and is classified as luminal-subtype breast cancer cell line (190).

The DU4475 cell line was derived from a 70 years old, Caucasian female patient with ductal carcinoma (190). The breast cancer cell line is characterized as a basal-A subtype and immunomodulatory (IM), and is also triple-negative (31).

The HCC1187 cell line was derived from a 41 years old, Caucasian, female patient who had received chemotherapy. The tumor was classified as stage IIA, grade 3 (T - tumor size, M – metastasis to regional lymph nodes, N – metastasis to distant lymph nodes (9)) invasive ductal carcinoma. HCC1187 is positive for the epithelial cell specific marker Epithelial Glycoprotein 2 (EGP2) and for cytokeratin 19. The cells are poorly differentiated and do not express the progesterone receptor (PR) or HER2-neu oncogene, but overexpresses p53 (190). The cell line is classified as basal-A subtype and IM, and is also triple negative (31).

The HCC1569 cell line was derived from a 70 years old, black female patient with a germline mutation in the FHIT gene, but had no family history of breast cancer. The patient had received prior chemotherapy. The tumor was classified as TNM stage IV, grade 3, metaplastic carcinoma with 4 out of 18 lymph node metastasis. HCC1569-cells are positive for expression of HER2-neu, negative for p53 and poorly differentiated. They are also positive for the epithelial cell specific marker EGP2 and cytokeratin 19, and negative for the expression of the estrogen receptor (ER) and PR by immunohistochemistry. This breast cancer cell line is classified as basal-A subtype (190).

The Hs578T cell strain was derived from a breast-carcinoma from a 74 years old, Caucasian female, along with the Hs578Bst (which is a normal fibroblast-like line from the same patient). Estrogen receptors are not expressed (190), and the cell line is classified as basal-B and is triple negative (31).

MA11 was derived from a 65-year-old Caucasian female, that originated as an invasive lobular carcinoma and metastasized to the bone marrow. The tumor was positive for hormone receptors ER and PR (191).

The MCF-7 breast cancer cell line was derived from a metastatic site (pleural effusion). The cancer originated as an adenocarcinoma in a 69-year-old Caucasian female. The MCF7 line has several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes, and is classified as luminal subtype. The cells also express the WNT7B oncogene, and genes for insulin growth factor binding proteins (45, 190).

MDA-MB-231 was derived from metastatic site (pleural effusion) of an adenocarcinoma. The patient was a 51 years old, Caucasian female. Cells express epidermal growth factor receptor (EGFR), transforming growth factor alpha receptor (TGF alpha), the WNT7B oncogene and shows mutations in TP53. This breast cancer cell line is classified as basal-B subtype, and is also triple negative (31, 190).

Table 1. Summary of cell lines, classification, expression of receptors, age of patient and growth conditions used in this paper (45, 190):

Cell line	Class	ER	PR	HER2	Triple negative	Age of patient (years)	Growht medium	Other conditions
AU565	Luminal	-	-	+		43	RPMI-1640 + 10% FBS	
MCF7	Luminal	+	+	-		69	DMEM + 10% FBS	Insulin 0,01 mg/ml
DU4475	Basal-A	-	-	-	+	70	RPMI-1640 + 10% FBS	Grows in suspension
HCC1187	Basal-A	-	-	-	+	41	RPMI-1640 + 10% FBS	
HCC1569	Basal-A	-	-	+		70	RPMI-1640 + 10% FBS	
Hs578T	Basal-B	-	-	-	+	74	DMEM + 10% FBS	Insulin 0,01 mg/ml
MA11	Basal-B	+	?			65	RPMI-1640 + 10% FBS	
MDAMB231	Basal-B	-	-	-	+	51	DMEM + 10% FBS	

DMEM: Dulbecco's Modified Eagle medium. RPMI: Roswell Park Memorial Institute medium. FBS: Fetal Bovine serum

2.2 Culture method

The cells were cultured according to the ATCC guidelines (Table 1), and subcultivated 1-3 times a week for optimal growth conditions. 5 I.U. of penicillin and 5 µg/mL of streptamycin were added to the complete growth medium to prevent bacterial growth. Incubation of the cells was done in culture conditions of 37°C and 5% CO_2.

2.3 Subculturing

Subculturing was carried out by removing the medium and washing the cells with 5-10 ml of Phosphate Buffered Saline (PBS, Sigma Aldrich). This was done to ensure the effect of trypsin, a serineprotease which breaks the peptide bonds, keeping the cells in aggregates and adherent to the culture-flask. After washing, 0,5-2 ml of 0,25% trypsin, 0,03% EDTA solution was carefully added. The flask was then allowed to sit at 37°C until the cells detached. Fresh culture medium was added to dilute the trypsin. The cells were then aspirated and dispensed into a new culture flask at a suitable subcultivation ratio, and the flask was refilled with complete growth medium to a volume of 10 ml, 15 ml or 25 ml to T-25, T-75- or T-175-flasks respectively.

Table 2. Summary of the different components used in cell culturing:

Chemicals	**Production company/contents**	**Experiment**
Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg glucose/L	Sigma-Aldrich®, St. Louis, USA	Methods 2.2 / 2.3
Roswell Park Memorial Institute Medium (RPMI-1640)	Sigma-Aldrich®, St. Louis, USA	Methods 2.2 / 2.3
Fetal Bovine Serum (FBS)	BIOCHROM AG	Method 2.2
Penicillin-Streptomycin	Sigma-Aldrich®, St. Louis, USA	Method 2.2
Trypsin-EDTA solution	Sigma-Aldrich®, St. Louis, USA	Method 2.3
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich®, St. Louis, USA	Method 2.3
Insulin	Sigma-Aldrich®, St. Louis, USA	Method 2.3

2.4 Protein Isolation

The medium was first removed and the cells washed with PBS like in subculturing (method 2.3). Trypsin was added and the cells sat until they detached, before aspirated in new medium (10-12 ml). The desired number of cells were then transferred to and grown on a 6 cm dish, after measuring and calculating the volume needed for the count of 300000 cells per dish; using this formula:

$$\textbf{Number of cells/dish (mL)}: \frac{Number\ of\ \frac{cells}{ml}(measured\ in\ aspirated\ cell - medium) \times X\ mL}{Desired\ number\ of\ cells}$$

After an incubation time of about 1-2 days (at 37°C, 5% CO_2), the cells were ready for protein isolation. First, the medium was removed and the cells were washed with PBS. Secondly, 150 µl of MKK lysis-buffer was added, and removed after about 1 minute. A scrape was used to transfer the lysate to an eppendorf-tube which was then left on ice for 15 minutes and vortexed twice (every 5 minutes) on a vortex during this time, before centrifugation (4°C) on maximum speed (13.000 rpm) for 10 minutes. The supernatant was transferred to a new eppendorf-tube.

2.5 Protein Assay

This assay was carried out according to the Bio-Rad DC Protein Assay protocol, to achieve the same amount of protein in each sample for western blot analysis. The assay is based on the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein (192).

2.6 Preparation of samples for western blot

Results from the protein assay were used to calculate the volume of cell-extract needed for each sample. MKK-lysis buffer was used as a filler to get a total volume of 20 µl in each sample. To each sample, LDS sample buffer (6 µl) and sample reducing agent (3 µl) was added (Table 3).

2.7 Immunoblotting (Western blot)

Immunoblotting (often referred to as western blotting) is a method for separating and visualizing proteins firstly by gel electrophoresis (Sodium dodecyl sulfate; SDS-page), and then by blotting on a nitrocellulose membrane (a solid support). The separated and immobilized proteins, transferred from the gel to the membrane, are then probed with an unlabeled specific antibody (polyclonal or monoclonal) to identify and quantitate any antigens present. After being probed with the primary antibody directed against the target protein, the membrane is washed and added a labeled secondary antibody (produced in a different species of animal, raised against the IgGs from the species of animal in which the primary antibody was raised). This results in detection of the present primary antibody, and thus the target protein. The results are then read on an infrared imaging system (193, 194).

The polyacrylamide 10 wells-gel (NuPAGE® Novex® Bis-Tris Mini gels, Invitrogen, Carlsbad, CA, USA) was transferred to a chamber filled with running buffer (Table 3). To each well there was added a sample of protein-lysate (25 µl), MagicMark™ XP standard (2µl) or SeeBlue®Plus2 Pre-stained standard (4 µl) (see Appendix II), after which the gel was run on the PowerEase® 500 (Invitrogen, NY, Life technologies™), on 200 V for 40 minutes.

The blotting materials consisted of a 7,5x7 cm nitrocellulose membrane (Oddyssey Nitrocellulose Membrane, LI-COR Bioscience), filter paper (8 x 8 cm), pads and blotting buffer (Table 3). The polyacrylamide gel was transferred to the membrane, which was then covered with a filter paper on each side. This "sandwich" was kept moist with blotting buffer, and held together by several pads in a blotting chamber, and run on the PowerEase®500 for 1,5 hours on 21 V.

Blocking was carried out by transferring the membrane to a centrifuge-tube containing 5 ml of commercial blocking buffer (Odyssey, Table 3) for the duration of 1 hour on a turning wheel. A dilution of the primary antibody (Table 3) with TWEEN® 20 and blocking buffer (1:500) was prepared in a BD-Falcon tube, and added to the membrane after discarding the commercial blocking buffer. This was further incubated on rotation for at least 12 hours. The membrane was then washed four times (5 minutes each) with TBS (Table 3), before adding the secondary antibody (Table 3), diluted in TWEEN® 20 and blocking buffer (1:20000). This was incubated for 1 hour wrapped in aluminum foil, followed by another round of washing with TBS.

The membrane was scanned using The Odyssey®Sa Infrared Imaging System. The results were gathered by the Odyssey®Sa Software pertaining to this imaging system.

Table 3.Summary of the different chemicals used in protein isolation and western blot:

Chemicals	**Production company/contents**	**Experiment**
MAPKK Lysis buffer	Made at the University of Tromsø (UiT) To 100 mL: 20 mM Tris (pH 7,0), 1% Triton X-100, 5 mM Tetra-sodium pyrophosphate (NaPPi), 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM vanadate (VO_4^{2-}), 0,27 M Sukrose, 10 mM β-Glycerophosphate, ad ddH_2O.	Methods 2.4, 2.5 and 2.6
Nupage® LDS sample buffer 4X	Invitrogen, NY, Life technologies™	Method 2.6
Nupage® Sample reducing agent	Invitrogen, NY, Life technologies™	Method 2.6
SeeBlue®Plus2 Pre-Stained Standard 1X	Invitrogen, NY, Life technologies™	Method 2.7
MagickMark™ XP Western Protein standard	Invitrogen, NY, Life technologies™	Method 2.7
NuPAGE® MES SDS Running Buffer (20X)	Invitrogen, NY, Life technologies™	Method 2.7
Blotting buffer	Contains: 29 g Tris base, 144 g glycine, 1 L methanol and ad 5 L H_2O (UiT)	Method 2.7

Odyssey Blocking buffer	LI-COR Bioscience	Method 2.7
Washing buffer Tris Buffered Saline (TBS, 10X)	Contains: 2 g KCl, 80 g NaCl, 40 mL Tris pH=7,5 (UiT)	Method 2.7
Primary antibody ERK3	ERK3 Abnova	Method 2.7
Primary antibody MK5	MK5-A7	Method 2.7
Secondary antibody	800 CW anti-mouse	Method 2.7
Primary antibody Actin	Anti-actin	Method 2.7
Secondary antibody	680 CW anti-rabbit	Method 2.7

2.8 A search for putative miRNA-targets

A search was carried out from different databases to discover putative miRNA targets for the potential binding sites in the 3'UTR-region of the genes *MAPK4* (ERK4), *MAPK6* (ERK3) and *MAPKAPK5* (MK5) (see Appendix I and III).

The first step was to find the official symbol belonging to the genes coding for the proteins ERK3, ERK4 and MK5. National Center for Biotechnology Information (NCBI) was used for this search (195).

Identification of miRNA was carried out from three different databases; TargetScanHuman (196), microRNA.org (197) and DIANA LAB (198). The results from these searches were gathered and compared to find common miRNA's. The target-sequence of the current miRNAs was then compared to the 3'UTR-fragment sequences used in the 3'UTR Lenti GoClones, as seen in Table 4.

2.9 miRNA mimics

miRNAs are implied to regulate protein-coding genes in a non-specific manner. They may act on all gene motifs that match their highly conserved seed site, giving miRNA-mediated regulation of genes and mRNAs several possible outcomes. In an experimental setting trying to silence a specific gene, one approach is to utilize synthetic miRNA-like RNA fragments called miRNA mimics. These fragments mimic endogenous miRNAs by having nucleotides

1-8 of their 5'end designed fully complementary to a motif in the 3'UTR of the target-gene. Potential binding to the target-gene will then cause translational inhibition, and because miRNA mimics have a reduced number of target sites compared to endogenous miRNAs, there is a lower risk of unwanted interference (139). We ordered the putative miRNA mimics from Shanghai GenePharma, to screen for miRNAs which could regulate the expression of the genes *MAPK4*, *MAPK6* and *MAPKAPK5*. The GenePharma miRNAs are chemically designed and optimized single-stranded nucleic acids, designed to mimic endogenous mature miRNAs entering directly into the miRNA processing pathway and having the same actions in cells (199). Our search for putative miRNA-targets (method 2.8) provided sense-sequences needed for ordering the desired miRNAs, summarized in Table 4.

Table 4. miRNA mimics used in transfection experiments (ordered from Shanghai GenePharma Co, Ltd):

Gene name	mRNA	miRNA	Sense-sequence (5' – 3')
MAPK4	ERK4	hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
		hsa-miR-922	GCAGCAGAGAAUAGGACUACGUC
		hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC
		hsa-miR-124-5p	CGUGUUCACAGCGGACCUUGAU
MAPK6	ERK3	hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
		hsa-miR-26b-5p	UUCAAGUAAUUCAGGAUAGGU
		hsa-miR-302a-5p	ACUUAAACGUGGAUGUACUUGCU
		hsa-miR-425	AAUGACACGAUCACUCCCGUUGA
		hsa-miR-1297	UUCAAGUAAUUCAGGUG
MAPKAPK5	MK5	hsa-miR-517-b	CCUCUAGAUGGAAGCACUGUCU
		hsa-miR-944	AAAUUAUUGUACAUCGGAUGAG
		hsa-miR-1284	UCUAUACAGACCCUGGCUUUUC
		hsa-miR-1305	UUUUCAACUCUAAUGGGAGAGA
		hsa-miR-548d-5p	AAAAGUAAUUGUGGUUUUUGCC
		hsa-miR-518d-5p	CUCUAGAGGGAAG0CACUUUCUG
		hsa-miR-559	UAAAGUAAAUAUGCACCAAAA

MAPK4: Mitogen activated protein kinase 4, ERK4: Extracellular signal-regulated kinase 4, *MAPK6*: Mitogen activated protein kinase 6, ERK3: Extracellular signal-regulated kinase 3, *MAPKAPK5*: MAPK activated protein kinase 5 (MK5)

2.10 Transfection of miRNA mimics

Transfection is a method for introducing functional genetic material into eukaryotic cells to study gene regulation and expression, cell-signaling, and is also used to inhibit or block the

expression of genes by siRNA. Lipofectamine 2000 is a cationic lipid reagent which is used (with a dedicated transfection medium like Opti-MEM) for transfecting genomic material into adherent cells. The cationic lipids interact with the negative charges of DNA, RNA or oligonucleotides, forming a compact structure. The condensed aggregates can interact with, and cross, the negatively charged cell membrane into the target cells due to the positive charge and lipophilic nature of cationic lipids (200).

HEK-293T cells, a highly transfectable derivative of human embryonic kidney 293 cells (ATCC® CRL-3216™) were used for transfection. The cells were cultured according to the ATCC guidelines, as described in methods 2.2 and 2.3, in DMEM (with 10% FBS, 5 I.U`s penicillin and 5 µg/mL streptomycin). Cells were plated in a 6-well plate, either 40000 cells per well and cultured for two days to a 70-90% confluence at the time of transfection, or plated at 110000 cells per well and reverse transfected simultaneously. Transfection was carried out according to the Invitrogen™ by Life Technologies™ protocol for Lipofectamine® 2000 Reagent (201).

2.11 Transfection by Lipofectamine® 2000 Reagent

3.75 µl of miRNA mimic (equivalent to 75 pmol) (see Table 4) was diluted in 250 µl Opti-MEM® medium in different test tubes, and added 250 µl of diluted Lipofectamine® Reagent in Opti-MEM® medium (3 µl in 250 µl respectively, per well). This mixture was incubated for 20 minutes in room temperature, before the aspirated plated cells were added 500 µl Opti-MEM® medium prior to carefully adding the RNA – lipid complex (500 µl) to each well. After a new incubation period of 4 – 6 hours at 37°C and 5% CO_2, 4 ml of complete growth serum was added to each well. This was further incubated for two days, at 37°C and 5% CO_2, before further analysis (201).

2.12 Reverse Transfection by Lipofectamine® 2000 Reagent

For each well, 3.75 µl of miRNA mimic (equivalent to 75 pmol) (see Table 4) was diluted in 250 µl Opti-MEM® medium in different test tubes, and added 250 µl of diluted

Lipofectamine® Reagent in Opti-MEM® medium (3 µl in 250 µl respectively, per well). This mixture was incubated for 20 minutes in room temperature, before carefully adding the RNA – lipid complex to the dispersed cells still in suspension, before they attached. The transfected cells were then incubated at 37 °C with 5% CO_2 for two days before further analysis.

2.13 FLOW Cytometry

The transfection efficiency was determined by using FLOW cytometry, which is a technique used for examining microscopic particles, like cells, in heterogeneous fluid-samples. The FLOW cytometer consists of a light-source, usually a laser, and detectors. Thousands of cells per second pass through the laser as a single file of particles, emitting light which is detected as forward scatter (by a detector in line with the laser, detecting light at a 20 degree angle), and side scatter (by a detector perpendicular to the laser, detecting light at a 90 degree angle). The scattering of light and fluorescence is detected as a signal (a current of photons) being amplified and converted to a voltage pulse. This voltage pulse gives information about particle size (from the intensity of the forward scatter), complexity and granularity (from the intensity of the side scatter), and more, over time. By producing unique light scatters the particles are sorted by characteristics, and the population of interest can be singled out by viewing the results on dual-parameter histograms (202).

Since reverse transfection is thought to increase cell exposure to transfection complexes because of increased amount of exposed cell surface (203) a fluorescent (FAM) – labeled miRNA was both transfected (method 2.11) and reverse transfected (method 2.12) in HEKT-293T cells (ATCC® CRL-3216™), and analyzed in a BD FACSCalibur flow cytometer (BD Biosciences, USA). Reverse transfection showed a higher transfection efficiency (see Figure 7 and appendix V), and this method (method 2.12) was used for further experiments.

2.14 Purification of total RNA

To study the effect of different miRNAs on the mRNA levels of ERK3, ERK4 and MK5, total RNA was purified after two days of incubation time from the cells transfected in method 2.12. This was done by using RNeasy Plus Mini Kit from Qiagen, according to the manufacturer`s

recommendations. After purification, the RNA was eluated with 40 µl of RNase free water. The concentration of RNA in the samples was measured with NanoDrop spectrophotometer from Thermo Fisher Scientific.

2.15 Reverse transcriptase quantitative PCR (RT-qPCR)

Quantitative PCR is used to detect and quantify gene transcripts. The samples were prepared for qPCR by converting the mRNA into complementary DNA (cDNA) through a reverse transcriptase (RT) reaction. Eurogentec Reverse Transcriptase Core Kit (Searing, Belgium) was used for this reaction, following the technical data sheet supplied. The volumes in the RT Reaction Mix were prepared to be sufficient for 400 ng of total RNA per 20 µl RT step, using random nonamers as primers. After the RT-reaction, cDNA was diluted in a ratio of 1:2 with RNase free water.

In real-time qPCR the amplification of a PCR product can be detected in a linear fashion over a range of PCR cycles. SYBR Green is a dye that has high affinity for double-stranded DNA (dsDNA) and low affinity for single-stranded DNA or RNA. When bound to dsDNA, the fluorescence yield from SYBR Green will increase with about 1000 fold, and increase proportionally with the DNA concentration, allowing real-time monitoring. Stratagenes MX3000P instrument was used to measure fluorescence.

Through the denaturation step, all cDNA becomes single stranded. In the annealing step, the primers will hybridize to the target sequence. Specific primers for each target gene (*MAPK4, MAPK6 and MAPKAPK5*) or reference gene (*ACTB* and *TFRC*) were added to the samples (summarized in table 5). The elongation phase enhances accumulation of dsDNA and the fluorescence intensity increases proportionally, determined by the initial concentration of the target.

The amplification plot reflects changes in fluorescence during cycling, and can be used to determine the initial copy number by defining the threshold cycle (Ct). Ct is the first cycle where the fluorescence can be statistically measured above background. This value has a direct correlation to the starting concentration, which means that high template - amounts in the sample yields low Ct-values. Since fluorescence only depends on presence of dsDNA, the specificity of the reaction is determined by the binding-degree of the primer to the template,

initiating DNA replication. The specificity of the primers was tested by generating a dissociation profile. The samples were subjected to a stepwise increase in temperature (from 55°C to 95°C), and fluorescence was plotted against temperature. The products will melt according to their composition, and give a drop in fluorescence due to dissociation of SYBR Green. So, if the samples are contaminated or contain primer-dimers or other non-specific products, their melting temperature is usually lower than that of the desired products. This will show as separate peaks in the dissociation plot (204, 205).

By using a mathematical formula called the comparative Ct – method (ΔΔCt – method), the relative amount of mRNA in each sample can be calculated:

$$\frac{Sample\ A}{\text{Sample B}} = \frac{2^{CtA\ index - CtA\ target}}{2^{CtB\ index - CtB\ target}} = 2^{\Delta\Delta Ct}$$

where Ct_{index} is the average of the Ct-values for the reference genes (*ACTB* and *TFRC*), and Ct_{target} is the Ct-value for the target gene (206).

cDNA equivalent to 40 ng of total RNA was amplified in a total of 25 µl of Brilliant II SYBR® Green PCR-mix (Agilent Technologies, California, USA) with 0,3 µM of forward and reverse primers (see Table 5). The primer for one of the reference genes (*TFRC*) was added in a ratio of 1:20 of the total PCR Master Mix volume of 25 µl (see Table 5). The PCR was run with the following conditions: 95°C for 15 minutes, following 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. To make sure that the samples did not contain genomic DNA, the setup included a NoRT – control (samples lacking the reverse transcriptase). To rule out contamination of the PCR Master Mix there was also included a NTC – control (no template control).

Table 5. Primer assays used in SYBR® Green-based expression analysis of individual genes:

Gene symbol	Primer-pair ID / Assay name	Production company
MAPK4 (ERK4)	H_MAPK4_1	Sigma-Aldrich®, St. Louis, USA
MAPK6 (ERK3)	H_MAPK6_1	Sigma-Aldrich®, St. Louis, USA
MAPKAPK5 (MK5)	H_MAPKAPK5_1	Sigma-Aldrich®, St. Louis, USA
ACTB	H_ACTB_1	Sigma-Aldrich®, St. Louis, USA
TFRC	Hs_TFRC_1_SG	QIAGEN

MAPK4: Mitogen activated protein kinase 4 (ERK4), *MAPK6*: Mitogen activated protein kinase 6 (ERK3), *MAPKAPK5*: MAPK activated protein kinase 5 (MK5), *ACTB*: β-actin, *TFRC:* Transferrin receptor.

2.16 Mission® 3'UTR Lenti GoClone™

To study a potential regulatory the effect of different miRNAs on the ERK3, ERK4 and MK5, HEK-293T cells were transduced with Mission® 3'UTR Lenti GoClone™ (SwitchGear Genomics) constructs harboring parts of the ERK3, ERK4 and MK5 3'UTRs fused to a luciferase reporter gene (RenSP) and a gene for puromycin resistance (Puro) (see appendix I). A wide range of mammalian cell lines can achieve stable expression due to the ability of the lentivirus backbone to infect several cell culture lines, overcoming low transfection difficulties. The viral particles are self-inactivating and replication incompetent, and the lentiviral constructs were delivered in HEK293T packaging cells. Each construct, in volumes of 0,2 ml with $\geq 10^5$ transducing units, was provided in DMEM with 10% heat inactivated FBS and penicillin-streptomycin. The Sigma-Aldrich® SwitchGear Genomics Mission® 3'UTR Lenti GoClone™ Technical Bulletin was followed as protocol (207).

On day 1, HEK-293T cells (ATCC® CRL-3216™) were plated (10000 cells per well) in a 24-well plate, in complete medium (DMEM with 10% FBS, 5 I.U`s penicillin and 5 µg/mL streptomycin). On day 2, the lentiviral stocks were thawed on ice. Hexadimethrine bromide (70 mg/ml) was added to the plated HEK-293T cells, at a final concentration of 8µg/ml, to enhance transduction, following addition of viral particles at a volume of 0,1ml ($\geq 5 \cdot 10^4$ transducing units). On day 3, the viral-particle containing medium was replaced with pre-warmed complete culture medium (DMEM with 10% FBS, 5 I.U`s penicillin and 5 µg/mL streptomycin). On day 5, the medium was replaced with fresh puromycin-containing medium at a concentration of 1,5 µg/ml, for puromycin selection of cells transduced with Mission 3'UTR Lenti GoClones. After 3 subsequent days the viral-particle containing cells were subcultured and dispensed into a 6-well plate, and further cultured in puromycin containing medium (1,5 µg/ml) (Sigma-Aldrich®, St. Louis, USA) until populations of puromycin-resistant cells were established. Stable integration was confirmed by Dual - Luciferase® Reporter Assay (method 2.17) before carrying out further experiments. A control was also included in this step, which contained only HEK-293T cells (no transducing units), but was added hexadimethrine bromide and cultured in the same puromycin-containing medium as the others. This was done to show that non-transduced cells did not show puromycin-resistance and would not survive.

Table 6. Summary of the Mission® 3'UTR Lenti GoClone™ used in experiments 2.16 and 2.17:

Gene name	**Cat. No.**	**Production company/contents/cat.no.**	**Experiment**
MAPK4	HUTR10812	SwitchGear Genomics, Sigma-Aldrich®, St. Louis, USA	2.13 and 2.14
MAPK6	HUTR08621	SwitchGear Genomics, Sigma-Aldrich®, St. Louis, USA	2.13 and 2.14
MAPKAPK5	HUTR03385	SwitchGear Genomics, Sigma-Aldrich®, St. Louis, USA	2.13 and 2.14
N.T. Control	HUTR003C	SwitchGear Genomics, Sigma-Aldrich®, St. Louis, USA	2.13 and 2.14

MAPK4: Mitogen activated protein kinase 4, *MAPK6*: Mitogen activated protein kinase 6, *MAPKAPK5*: MAPK activated protein kinase 5, N.T. Control: Non Target Contro

2.17 Dual - Luciferase® Reporter Assay System

To study the gene expression of *MAPK4*, *MAPK6* and *MAPKAPK5* transfected with selected miRNAs, we applied the Dual-Luciferase® Reporter (DLR™) Assay System where two individual reporter enzymes are measured simultaneously within a single system. The main reporter, in this case *Renilla* luciferase, correlates with the gene expression of the target-genes. *Renilla* luciferase is a 36 kDa monomeric protein and functions as a genetic reporter right after translation. The control reporter, in this case Firefly luciferase, serves as an internal control. Firefly luciferase is a 61 kDa monomeric protein that in the same way as *Renilla* luciferase has enzymatic activity without post-translational processing. Both yield luminescence by photon emission through oxidation, but can be discriminated between because of their different enzyme structures and the substrates needed for their bioluminescent reactions (208).

The cells containing stably transduced Mission 3'UTR Lenti GoClones (from method 2.16) were plated out in 6-well plates, as described in method 2.10, with 110000 cells per well and 1,5 µg/ml puromycin-containing medium (as used in method 2.16). Included in the experiment was a control vector construct with a non-specific 3'UTR, containing non-conserved, non-genic and non-repetitive human genomic fragments. Each construct was then reverse transfected with the corresponding miRNAs (see Table 4) as described in method 2.12. After two days the cells were lysed by adding diluted Passive Lysis 5x Buffer (Promega), which gives rapid lysis of cultured mammalian cells without scraping.

The growth medium was removed from the cultured cells, and washed with phosphate buffered saline (see Table 2). The rinse was completely removed before adding 500 µl of 1xPLB (3 ml Passive Lysis 5x Buffer diluted in 12 ml distilled water) to each well. The lysis buffer was allowed to work for 15 minutes on a rocking platform in room temperature, before transferring the lysates into individual tubes. Lysate samples were cleared for 30 seconds at top speed in a refrigerated micro-centrifuge, and 20 µl of each sample was transferred to individual wells in a 96-well plate. Following the Dual-Luciferase® Reporter Assay Protocol, 100 µl Luciferase Assay Reagent II (LAR II) was dispensed by a reagent injector into each sample-well. This measures the firefly luciferase, and generates a stabilized luminescent signal. Straight after the firefly reaction is quenched, the *Renilla* luciferase reaction is initiated by the reagent injector adding 100 µl Stop & Glo® Reagent into the same sample-wells,

giving stabilized luminescence from the *Renilla* luciferase. Rapid quantitation of both reporters yields linear assays, and luminescence was detected in the Thermo Scientific™ Luminoskan Ascent Microplate Luminometer (Thermo Scientific). Ascent Software for Luminoskan (version 2.6) was used for collecting the results (208).

3 Results

3.1 Optimization of miRNA transfection efficiency

In experiments directed at miRNA target identification, it is important to use conditions that result in highest transduction efficiency, to maximize the level of inhibition on mRNA and/or protein expression. To investigate the influence of two different transfection methods on tranfection efficiency, FAM™- Dye Labeled miRNA-mimics and negative control miRNA-mimics were transfected by using Lipofectamine® 2000 reagent (method 2.11 and 2.12) in HEK-293T cells (ATCC® CRL-3216™). The FAM™ Dye is a fluorescent label that has been extensively tested in human cell lines and tissues and validated to produce no identifiable effects on known miRNA function. It is used for monitoring transfection efficiency in transfection experiments and enables direct observation of cellular uptake.

Both conditions resulted in the presence of a fluorescent signal in a high percentage of cells and no cytotoxicity was observed after 48 hours. The results from the flow-cytometry (method 2.13) showed a pronounced difference in transfection efficiency when comparing regular transfection (method 2.11) and reverse transfection (method 2.12) as seen in Figure 7. The average positive transfected cells were almost 100% with reverse transfection, compared to an average of almost 67% with regular transfection.

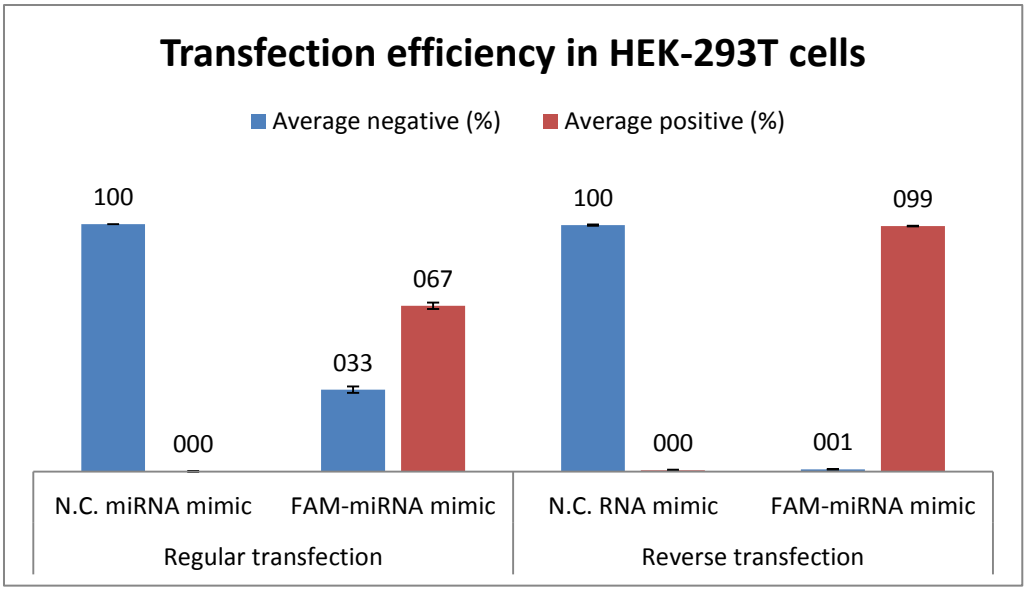

Figure 7. Flow cytometry to evaluate transfection efficiency of miRNA-mimics in HEK-293T cells comparing regular- and reverse transfection. N.C. miRNA mimic: negative control miRNA mimic (not labeled by fluorescent dye) is compared to FAM-miRNA mimic: miRNA-mimic labeled by fluorescent dye using two different methods of transfection.

3.2 MK5 and ERK3 are expressed in breast cancer cell-lines

The mitogen-activated protein (MAP) kinase pathways have been implicated to play an important role in many cancers, including breast cancer (209). Tumorigenic processes may also involve the atypical MAP kinases ERK3/4 and MK5 and studies have shown that these kinases can participate in several processes that are deregulated in cancer, including cell proliferation, cell motility, invasiveness, and angiogenesis (60, 61, 210, 211)**.** The *ras*, *raf* and *c-myc* genes are commonly mutated in cancer, and ERK3/4 and MK5 seem to act downstream of the Ras/Raf pathway and are also implicated in the regulation of c-Myc expression. However, the precise molecular mechanisms of ERK3, ERK4 and MK5 in cancer and cancer-related signaling pathways remains to be unraveled (209).

There is little knowledge surrounding regulation of the atypical MAP kinases, and their correlation to breast cancer. To investigate and compare the relative expression level of ERK 3 (~ 100 kDa) (212), and its substrate MK5 (~ 54kDa) (64), we used the method of western blotting analysis in 8 different breast cancer cell lines (BCCL) belonging to all three molecular subtypes. Due to insufficient binding by the available antibodies, it is not possible

to detect ERK4 by a regular western blot, so this protein was excluded from these initial analyses.

In order to ensure equal loading, the blots were also probed with anti-actin antibody, however the expression-level of actin in the different breast cancer cell lines varied far more than expected (see appendix VI) considering that a protein assay had been conducted prior to the western blot analysis to achieve the same amount of protein in each sample. Since we could not rule out the possibility of different levels of actin in our cell lines (which is a well-known phenomenon (213)), we chose to compare the integrated intensities from each cell line directly, for both the MK5 and ERK3 blots.

Results of the western blot analysis (figures Figure 8 and Figure 9) show that both ERK3 and MK5 are present in all the breast cancer cell lines, however the expression level varies greatly and does not correlate with their molecular subtype. The western blot also shows the presence of two different bands located at around 80 – and 100 kDa for ERK3 and two bands at around 54- and 70 kDa for MK5. The bands at around 80- and 70 kDa are probably caused by unspecific binding by the antibodies in the ERK3 and MK5 experiments respectively.

Our results confirm the presence of ERK3 and MK5 in all breast cancer cell lines tested, however little is known about the ERK3, ERK4 and MK5 protein levels in breast cancer (and in cancers in general), and it remains to be established whether aberrant expression of these proteins contributes to oncogenesis.

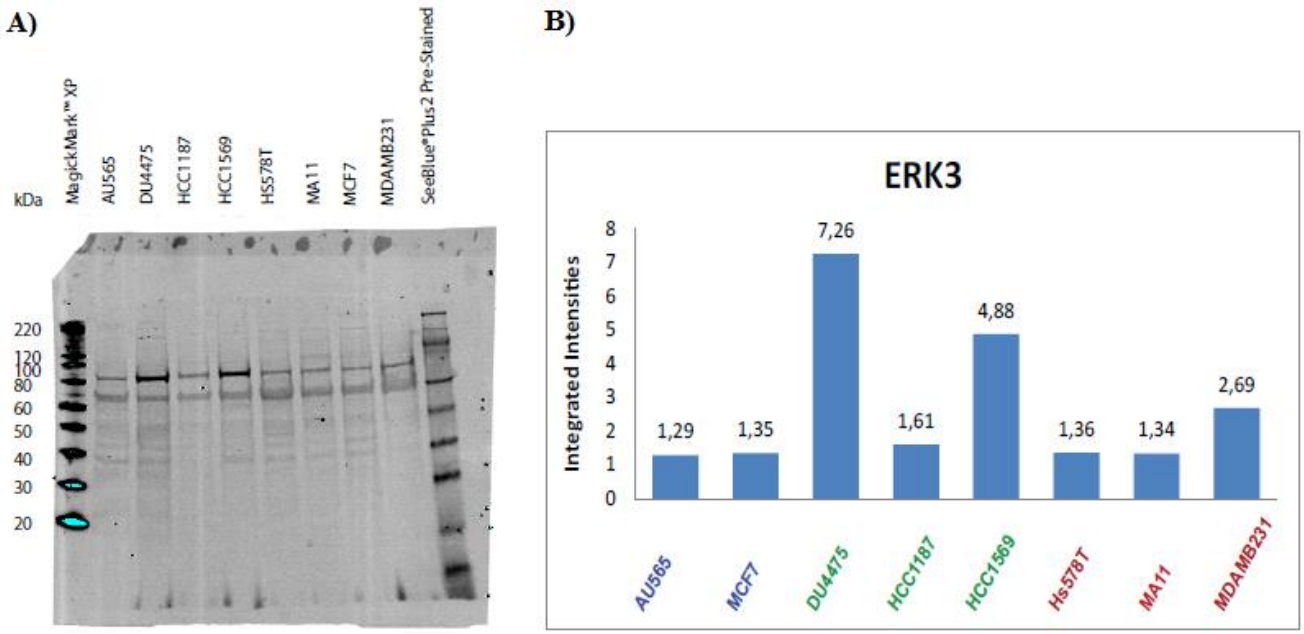

Figure 8. Expression of ERK3 in breast cancer cell lines. 300000 cells/dish were grown in a 6-cm-dish for 1-2 days before they were lysed. Proteins were measured in a protein assay prior to western blot analysis. ERK3 Abnova (1:500) was used as primary antibody and 800 CW anti-mouse (1:20000) was used as secondary antibody. A) Western Blot of the ERK3 protein expression in different breast cancer cell lines. B) Quantification of the western blot show variations in the expression of ERK3 proteins between different breast cancer cell lines, classified into subtypes by color: Luminal: blue, Basal-A: green, Basal-B: red.

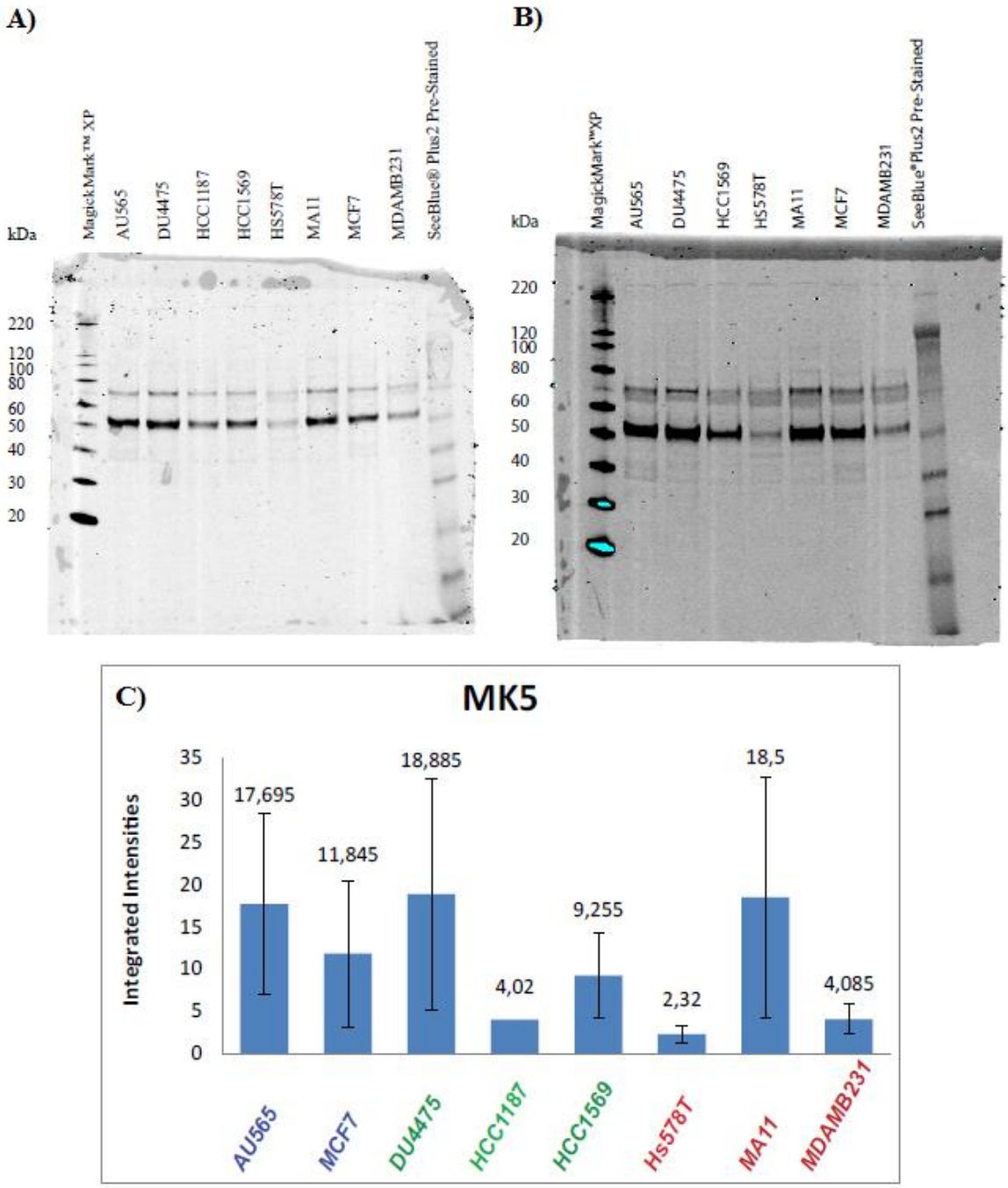

Figure 9. Expression of MK5 in breast cancer cell lines. 300000 cells/dish were grown in a 6-cm-dish for 1-2 days before they were lysed. Proteins were measured in a protein assay prior to western blot analysis. MK5-A7 (1:500) was used as primary antibody and 800 CW anti-mouse (1:20000) was used as secondary antibody. A) Western Blot of the MK5 protein expression in different breast cancer cell lines. In this western blot, the cell line MDAMB231 is not comparable due to a low protein count. B) Western blot with the samples from blot A, except for a new protein assay of MDAMB231, to achieve a comparable result. This immunoblot is missing the cell line HCC1187 due to small sample size. Quantification of the western blot show variations in the expression of the MK5 C) Quantification of the western blots shows variations in the expression of MK5 between the different breast cancer cell lines, classified into subtypes by color: Luminal: blue, Basal-A: green, Basal-B: red. The column-diagram is showing the combined integrated intensities of blot A) and B), with SEM.

3.3 The mRNA levels of ERK3/4 and MK5 are not significantly affected by miRNAs predicted to bind in their 3'UTR

Regulation of cellular signaling pathways, such as the mitogen-activated protein (MAP) kinase pathways, involves miRNAs. These small RNA-molecules silence genes at either transcriptional or translational levels, often by targeting their 3'-untranslated regions. A single miRNA can be predicted to target several hundred genes, and about 60% of mRNAs have predicted binding sites for one or multiple miRNAs in their 3'UTR (92, 126, 214). The function of a miRNA is ultimately defined by the genes it targets and its effects on their expression. In cancer cells and breast cancer cell lines there seems to be an abnormal expression of miRNAs, and such an imbalance is closely related to malignant proliferation and migration of cancer cells. miRNAs may act as oncogenes or tumor suppressors, but their exact role in specific steps of malignant progression is not yet known (215).

As mentioned in part 3.2, little is known about the regulation and function of ERK3/ERK4. It has been shown that both ERK3 and ERK4 can activate MK5. MK5 in turn, phosphorylates transcription factor FOXO3a, which leads to an increased level of mir34b/c. mir34b/c inhibits the cell cycle and prevents translation of c-*myc* mRNA (211). However, currently no other miRNAs have been linked to the regulation of this ERK3/4-MK5 atypical MAP kinase signalling pathway. A number of freely available programs predict potential mRNA targets for individual miRNAs in silico. However the predictions must be confirmed using miRNA target validation techniques that aim to detect changes in mRNA and/or protein level in response to miRNA overexpression.

In order to investigate if the expression of eitherERK3, ERK4 or MK5 may be regulated by miRNAs, we selected 16 different miRNAs with potential binding target sites (predicted by the TargetScanHuman, microRNA.org and DIANA LAB bioinformatics approaches) in the 3'UTR of the mRNAs (see Table 7). Since miRNA target silencing can occur both at the level of translation and mRNA degradation, we aimed to investigate if the expression of our selected miRNAs would affect the mRNA levels of ERK3, ERK4 and MK5 in HEK-293T cells by using real-time RT-(q)PCR.

Table 7. miRNA mimics used in transfection experiments (ordered from Shanghai GenePharma Co, Ltd):

Gene name	mRNA	miRNA
MAPK4	ERK4	hsa-miR-423-5p
		hsa-miR-922
		hsa-miR-34c-5p
		hsa-miR-124-5p
MAPK6	ERK3	hsa-miR-26a-5p
		hsa-miR-26b-5p
		hsa-miR-302a-5p
		hsa-miR-425
		hsa-miR-1297
MAPKAPK5	MK5	hsa-miR-517-b
		hsa-miR-944
		hsa-miR-1284
		hsa-miR-1305
		hsa-miR-548d-5p
		hsa-miR-518d-5p
		hsa-miR-559

MAPK4 is the gene for which the mitogen activated protein kinase 4, ERK4, is encoded. *MAPK6* it the gene for which mitogen activated protein kinase 6, ERK3 is encoded and *MAPKAPK5* is the gene for which MAPK activated protein kinase 5, MK5 is encoded.

The HEK-293T cells were transfected with the miRNAs with potential binding properties for ERK3, ERK4 and MK5 mRNA 3'UTRs (see Table 7), and negative control miRNA (N.C. miRNA) without binding-potential. We also included non-transfected HEK-293T cells (N.T. HEK). Two days after transfection, total RNA was extracted from the HEK-293T cells (three biological replicates per mimic miRNA) using the RNeasy Plus Mini Kit from Qiagen, according to the manufacturer's recommendations. First strand cDNA synthesis was done on 400 ng total RNA per reaction using the Reverse Transcriptase Core Kit (Eurogentec) and mRNA levels were quantified using qRT-PCR with Brilliant II SYBR® Green PCR-mix (Agilent Technologies) on Stratagenes Mx300P QPCR System Instrument (Agilent Technologies), with normalization of gene expression to the reference (housekeeping) genes *ACTB* and *TFRC*.

Figure 10 shows the relative amount of ERK4 mRNA after reverse transfection of miR-423-5p, miR-922, miR-34c-5p and miR-124-5p, related to the negative control miRNA (N.C.

miRNA). No significant effect of any of the four miRNAs was observed on the levels of ERK4 mRNA (MAPK4 mRNA).

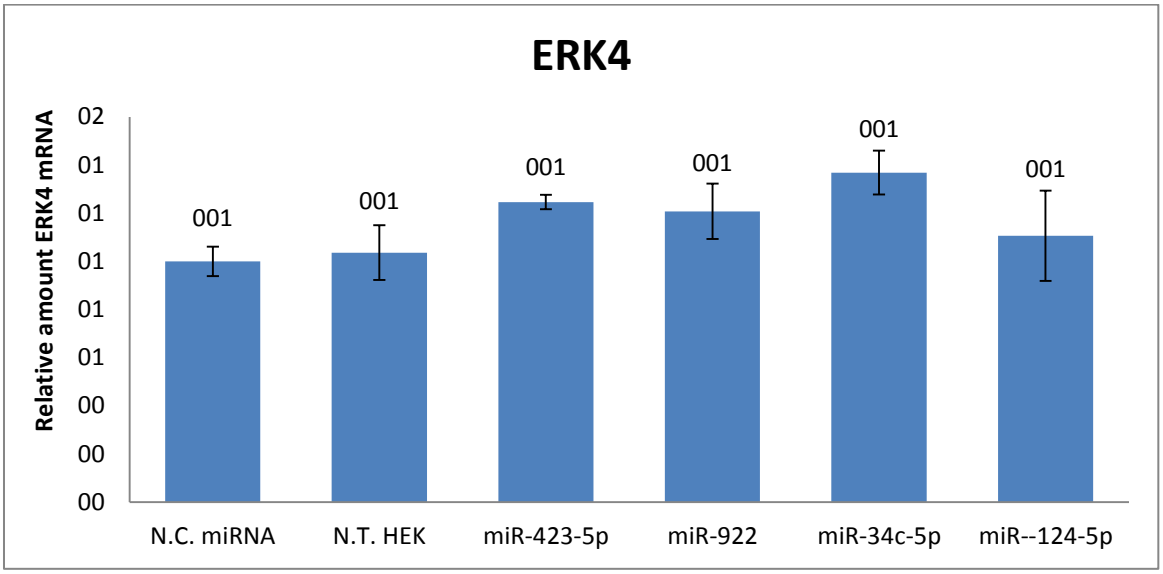

Figure 10. Expression of ERK4 mRNA. RT-(q)PCR analysis of ERK4 mRNA expression in HEK-293T cells transfected with four different miRNAs with potential binding target sites in the 3'UTR of the ERK4 mRNA. Expression levels of mRNA was determined in individual samples by RT-qPCR. Fold changes compared with the internal controls were calculated based on the ΔΔCt – method. Data represented as the means ± SEM.

Figure 11 shows the relative amount of ERK3 mRNA after reverse transfection of miR-26a-5p, miR-26b-5p, miR-302a-5p, miR-425 and miR-1297, related to the negative control miRNA (N.C. miRNA). Although the standard deviation of the negative control miRNA is substantial, the mean value is similar to the second internal control (non-transfected HEK-293T cells) and was considered to still serve as the relating control. We observed no significant effect of any of the five miRNAs on the levels of ERK3 mRNA (MAPK6 mRNA).

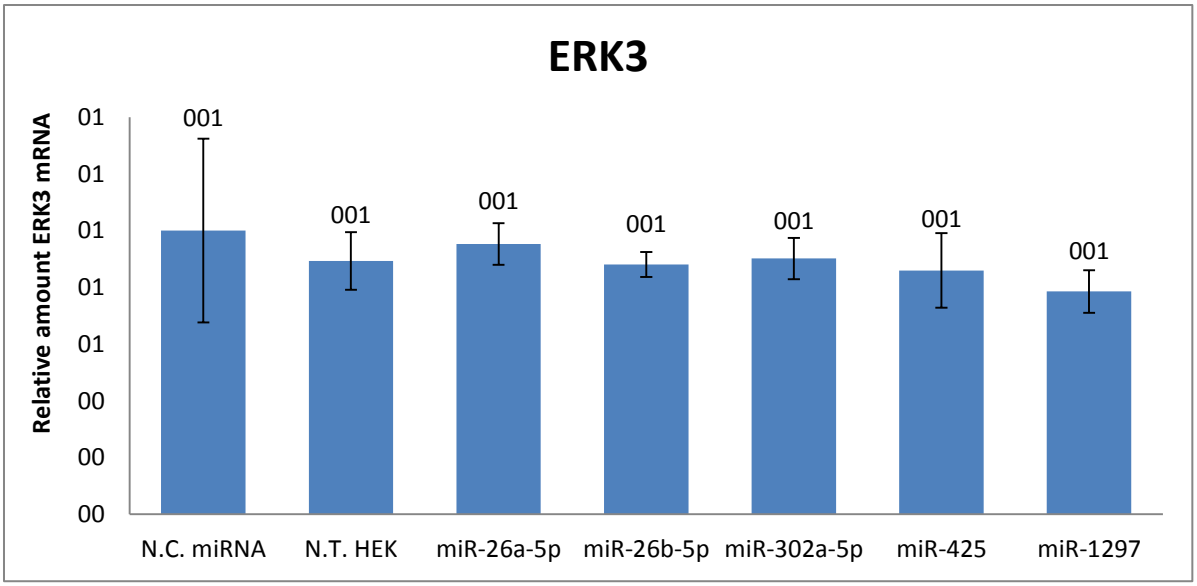

Figure 11, Expression of ERK3 mRNA. RT-(q)PCR analysis of ERK3 mRNA expression in HEK-293T cells transfected with five different miRNAs with potential binding target sites in the 3'UTR of the ERK3 mRNA. Expression levels of mRNA was determined in individual samples by RT-qPCR. Fold changes compared with the internal controls were calculated based on the ΔΔCt – method. Data represented as the means ± SEM.

Figure 12. A shows the relative amount of MK5 mRNA after reverse transfection of miR-517-b, miR-944, miR-1284 and miR-1305, related to the negative control miRNA (N.C. miRNA). Figure 12.B shows the relative amount of MK5 mRNA after reverse transfection of miR-548d, miR-518-d and miR-559 related to the N.C. miRNA. Here, no significant change of the MK5 mRNA level was observed by any of the miRNAs with potential binding target sites in the MK5 3'UTR.

The results from these experiments show that neither of the miRNAs with in silico predicted target sites in the 3'UTR of each of the three atypical MAPKs (summarized in Table 7) caused a significant decrease in cellular mRNA level of these kinases. However, this does not rule out the possibility of regulation through translational repression.

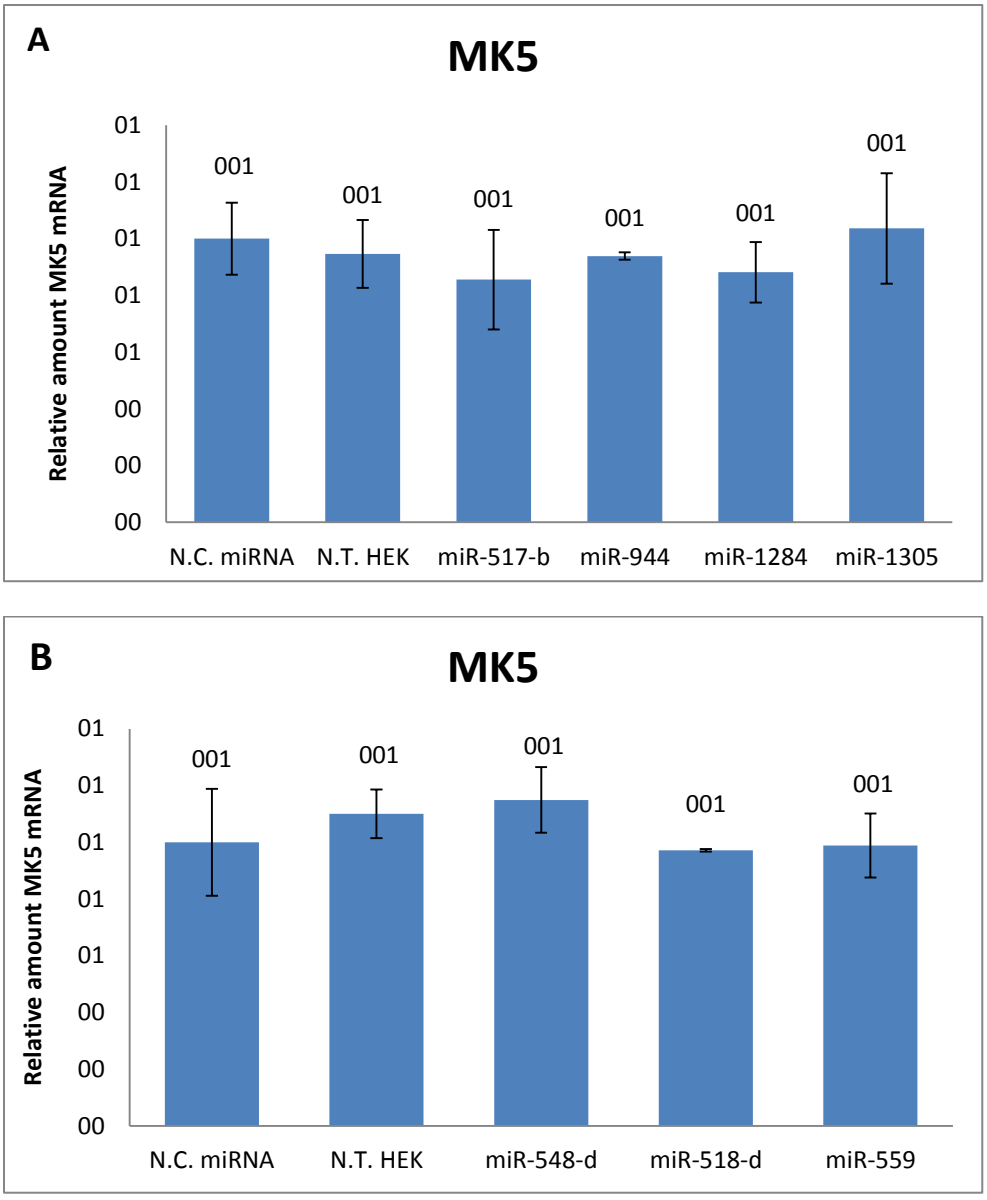

Figure 12. Expression of MK5 mRNA. RT-(q)PCR analysis of MK5 mRNA expression in HEK-293T cells transfected with seven different miRNAs with potential binding target sites in the 3'UTR of the MK5 mRNA in two separate experiments (A and B). Expression levels of mRNA was determined in individual samples by RT-qPCR. Fold changes compared with the internal controls were calculated based on the ΔΔCt – method. Data represented as the means ± SEM.

3.4 A potential regulation of ERK3 and MK5 protein levels by miRNAs predicted to bind in the ERK3 and MK5 3'UTR

If a given mRNA is a true target of a specific miRNA, then change in the miRNA expression should correspond to a predictable change in the amount of protein encoded by the target mRNA. The most commonly used approach to verify a miRNA target site is by linking the mRNA target to a luciferase reporter. A change in luciferase protein level will indicate whether a miRNA can bind to the 3'UTR and regulate the expression of the gene at the protein level.

To determine if any of our selected miRNAs act to regulate ERK3, ERK4 or MK5 at the level of translation, a renilla luciferase reporter assay was conducted (method 2.17). Prior to the experiments, we established four different HEK293T cell lines containing viral vectors (Lenti GoClone) that stably express a luciferase reporter gene (RenSP) fused to a part of the ERK3-, ERK4- or MK5 3'UTR sequences, and a positive control vector lacking a 3'UTR sequence (P.C. construct) in method 2.16.

The stably transfected cell lines containing the ERK3-, ERK4- or MK5 3'UTR fused to RenSP were reverse transfected with the pertaining miRNAs, including a negative control miRNA (N.C. miRNA). We also included the cell line harboring the positive control vector (P.C. construct) and a non-transfected control (N.T. control) for each of the three experiments (method 2.12). Due to limited time only one biological replicate, and two technical replicates was carried out. As a result of extremely large variations within each sample-replicate in parts of the experiment, the first technical replicate was not included in these results.

Figure 13. shows the relative luciferase activity in the HEK293Tcells expressing ERK4 3'UTR- RenSP, 48 hours after reverse transfection with miR-423-5p, miR-922, miR-34c-5p and miR-124-5p. As seen in the figure, there is no significant change in the relative luciferase activity in the transfected constructs compared to the controls for any of the miRNAs. This clearly indicates that none of the chosen miRNAs binds to this part of the ERK4 3'UTR and regulate ERK4 protein expression.

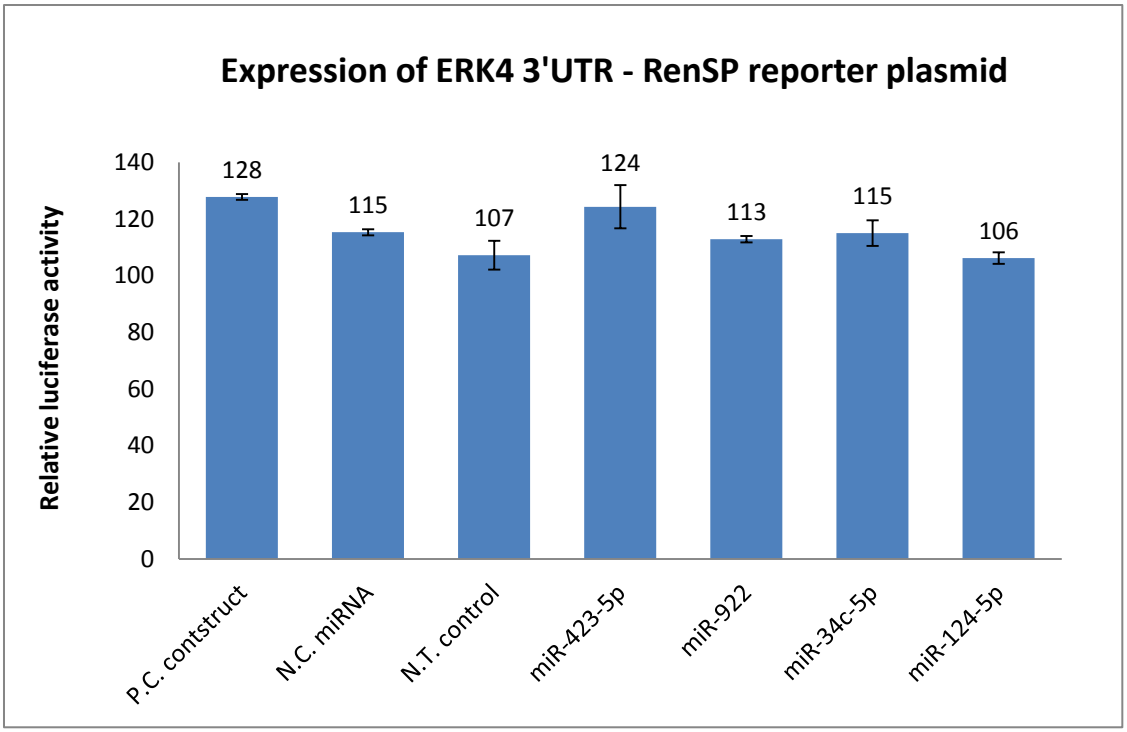

Figure 13. Relative luciferase activity in HEK293T cells expressing ERK4 3'UTR- RenSP reporter plasmids transfected with pertaining miRNAs or negative control miRNA (N.C. miRNA), no miRNA (N.T. control) or positive control vector (P.C. construct). Luciferase activity was measured 48 h transfection. Values are presented as mean relative luciferase activity ± SEM.

Figure 14. shows the relative luciferase activity in the HEK293Tcells expressing ERK3 3'UTR- RenSP 48 hours after reverse transfection with miR-26a-5p, miR-26b-5p, miR-302a-5p, miR-425 and miR-1297. Due to huge variations in the relative luciferase activity in the N.T control and a fairly low relative luciferase activity in the N.C miRNA control, it is difficult to draw any firm conclusions from this experiment. If we choose to relate the miRNAs with potential binding sites in ERK3 3'UTR to the N.C miRNA, the expression of miR-26a-5p, miR-425 and miR-1297 seems to cause an up-regulation of the luciferase expression. However, if we choose to relate the relative luciferase expression to the positive control vector (lacking a 3'UTR sequence, P.C. construct), the expression of miR-26b-5p and miR-302a-5p cause a down-regulation of the luciferase expression. Since the relative luciferase activity of the cells transfected with miR-26a-5p, miR-425 and miR-1297 are comparable to that of the P.C. construct, it seems more likely that the correct interpretation of these results is a potential binding to the ERK3 3'UTR by miR-26b-5p and miR-302a-5p causing a subsequent down-regulation of the luciferase expression.

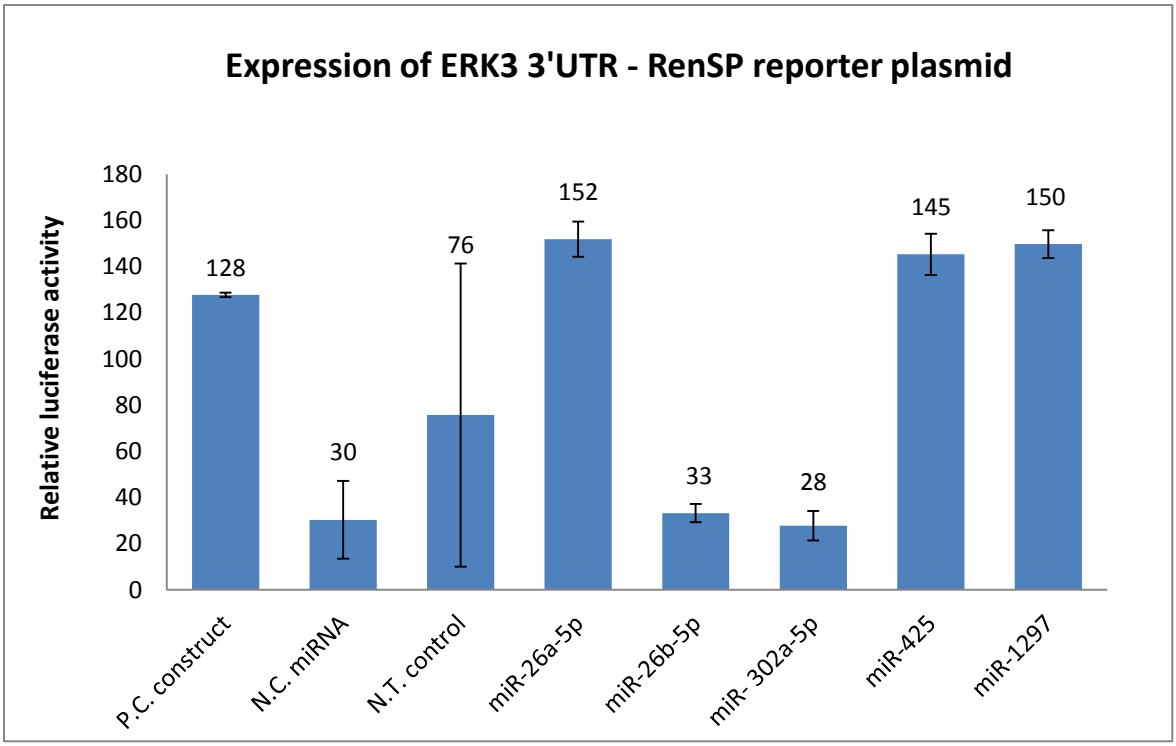

Figure 14. Relative luciferase activity in HEK293T cells expressing ERK3 3'UTR- RenSP reporter plasmids transfected with pertaining miRNAs or negative control miRNA (N.C. miRNA), no miRNA (N.T. control) or positive control vector (P.C. construct). Luciferase activity was measured 48 h after transfection. Values are presented as mean relative luciferase activity ± SEM.

Figure 15. shows the relative luciferase activity in the HEK293Tcells expressing MK5 3'UTR- RenSP 48 hours after reverse transfection with miR-517-b, miR-944, miR-1284, miR-1305, miR-548d, miR-518-d and miR-559. Due to a substantial difference in the relative luciferase activity between the negative control miRNA (N.C. miRNA) and the non-transfected control (N.T. control), which had a quite low luciferase activity, it is also difficult to draw any firm conclusions from these results. Since the relative luciferase activity for the N.C. miRNA is similar to the luciferase activity of the positive control vector (P.C. construct), it seems more likely that the N.C. miRNA and P.C. construct represent the correct measurements, while the N.T. control may have been compromised in the experimental process or during the luciferase assay.

If we choose to relate the miRNAs with potential binding sites in MK5 3'UTR to the N.C miRNA and the P.C. construct, the expression of miR-1305 seems to cause a slight up-regulation of the luciferase expression. In contrast, the expression of miR-517-b, and miR-548d-5p, miR-518-d-5p and miR-559 appear to cause a down-regulation of the luciferase expression. Cells transfected with miR-944 and miR-1284 have comparable luciferase

expressions to that of the N.C. miRNA and the P.C. construct, underscoring the probability that the correct interpretation of these results is a potential binding of miR-517-b, and miR-548d-5p, miR-518-d-5p and miR-559 to the MK5 3'UTR.

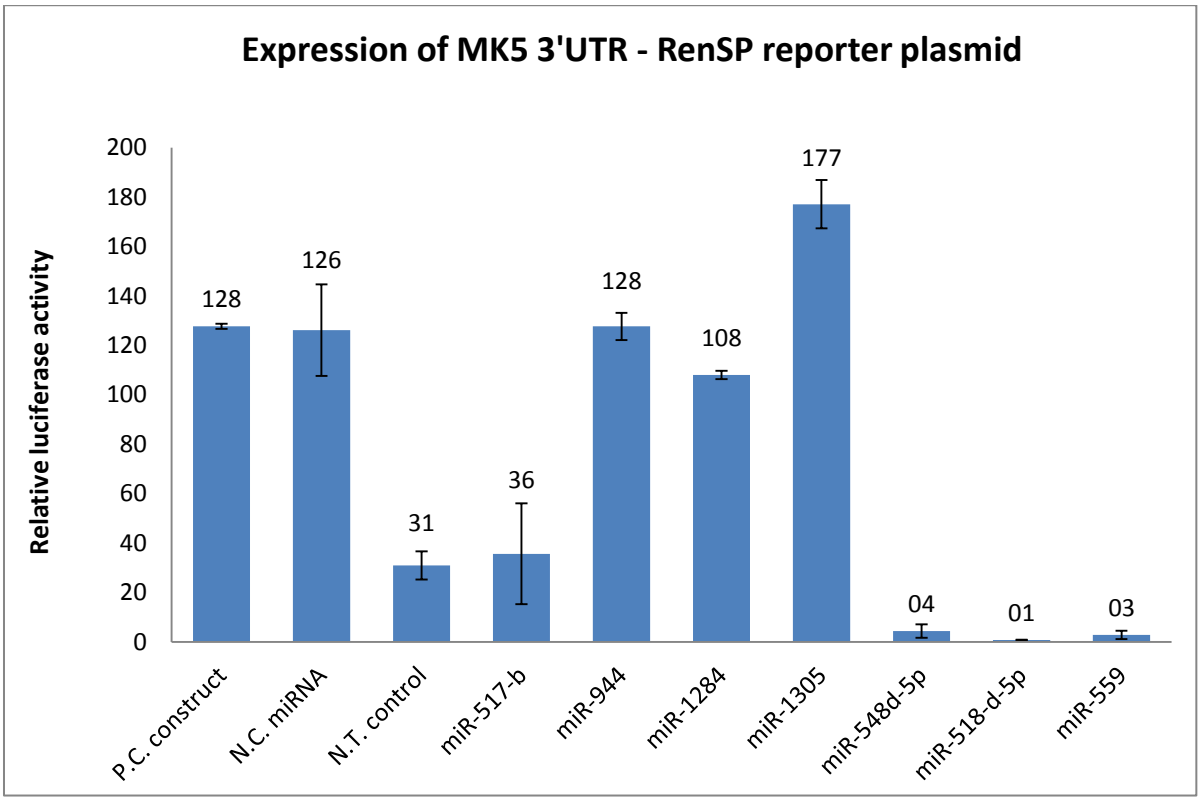

Figure 15. Relative luciferase activity in HEK293T cells expressing MK5 3'UTR- RenSP reporter plasmids transfected with pertaining miRNAs or negative control miRNA (N.C. miRNA), no miRNA or positive control vector (P.C. construct). Luciferase activity was measured 48 h after transfection. Values are presented as mean relative luciferase activity ± SEM.

3.5 A potential correlation between the expression of miRNA predicted to bind in the 3'UTR of ERK3 and MK5, and ERK3 and MK5 protein expressions in different subtypes of breast cancer cell lines.

Although the ERK3/4-MK5 pathway has been implicated in processes involved in cancer development and progress, in general there is not much knowledge surrounding these atypical protein kinases, as to how they are regulated or what their targets are (209).

The diverging expression of developmental genes is often seen as a cause of diseases, such as cancer (184). In the regulation of cancer development there seems to be a common pattern shared by miRNAs and protein oncogenes and tumor suppressors (216, 217), but the relationship between cell signaling pathways and the miRNA pathway is not well understood (218). A report from 2009 described a connection between miRNA expression and the Raf/MAPK/Erk pathway, suggesting a potential regulation by the let-7 miRNA-family (219). Another study has also shown a connection between the miRNA-generating complex and the MAPK/Erk pathway. The authors found that to effect mitogenic signaling, the MAPK/ERK kinases act on the miRNA-generating complex by phosphorylating the transactivating response RNA-binding protein (TRBP), participating in Dicer-mediated cleavage of the pre-miRNA. This phosphorylation lead to enhanced stability of the miRNA-generating complex, and resulted in an increased miRNA production and miRNA-mediated gene silencing. They suggested that through parallel mechanisms, the Raf/MAPK/Erk cascade affect cell signaling by regulating the miRNA expression (218).

Having established that the ERK3 and MK5 atypical kinases are expressed in all breast cancer cell lines tested, we wanted to search for a potential connection between the expression of our previously selected miRNAs and the protein expression in a biologically relevant system. To identify a potential correlation between the expression of the selected miRNAs predicted to bind in the 3'UTR of ERK3 and MK5, (see Table 7) we performed an in silico analysis of the expression selected miRNAs (previously obtained by small RNA profiling by Applied Biosystem's next-generation sequencing system (SOLiD5500™ XL)) and related that expression to the pertaining expression of ERK3 and MK5 in the same cell lines (obtained by western blot analysis).

Figure 16 shows the expression of all the miRNAs with predicted binding sites in the 3'UTR of ERK3, ERK4 and MK5. It seems as these miRNAs belong to one of three groups: those that generally are expressed at a high level in all the breast cancer cell lines (BCCL) (miR-26a, miR-26b and miR-425). miRNAs expressed, but not highly expressed, in all or most BCCL (423-5p, miR-34c-5p, miR-517-b, and miR-548d-5p) or miRNAs that show a very low or no expression in any of the cell lines (miR-922, miR-124-5p, miR-302a-5p, miR-1297, miR-944, miR-1284, miR-1305, miR-518d-5p, and miR-559). This miRNA expression pattern can probably be explained by the fact that miRNAs are highly tissue specific with highly variable expression patterns comparing different tissues (220).

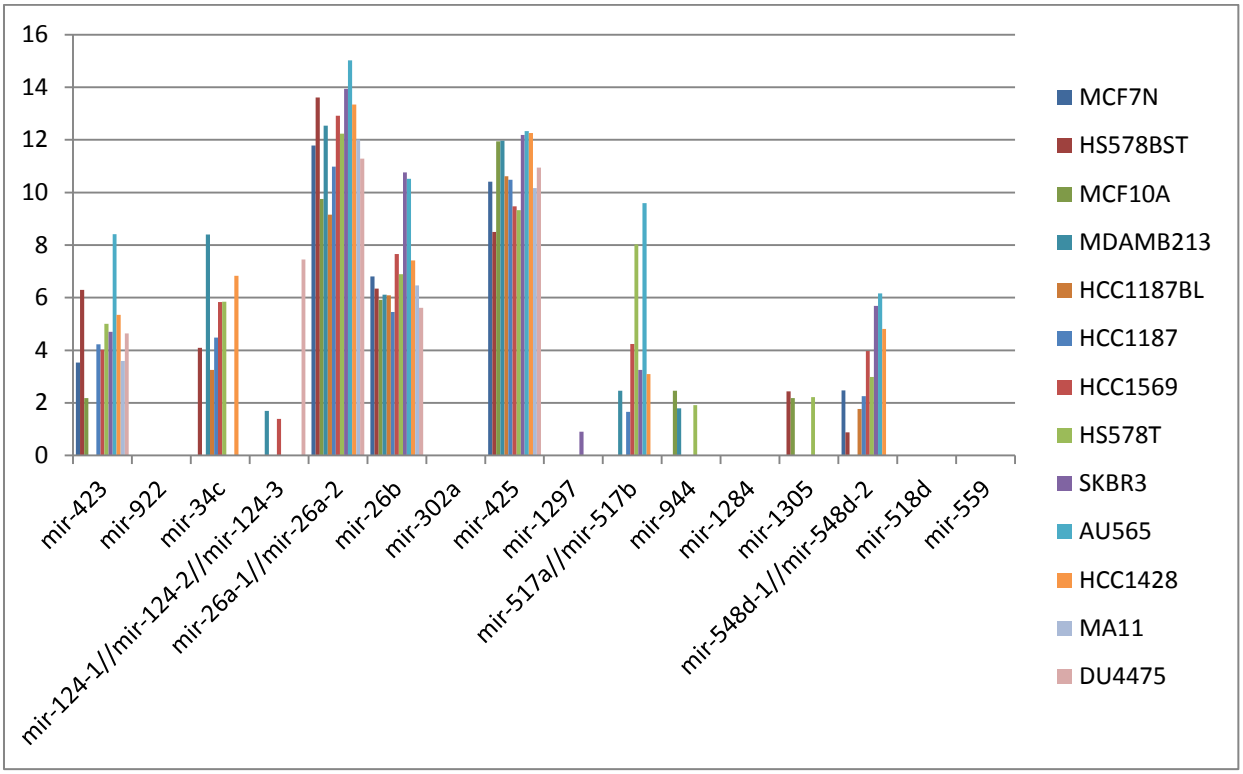

Figure 16. The expression of selected miRNAs (see Table 7) in the different breast cancer cell lines. The tendencies can be divided into three categories; 1) highly expressed in all BCCL, 2) expressed in most BCCL and 3) low/no expression in the BCCL.

In Figure 17 we show a comparison between the expression of the most highly expressed miRNAs for each kinase (ERK3 and MK5), against the expression of ERK3 and MK5 in the same breast cancer cell lines.

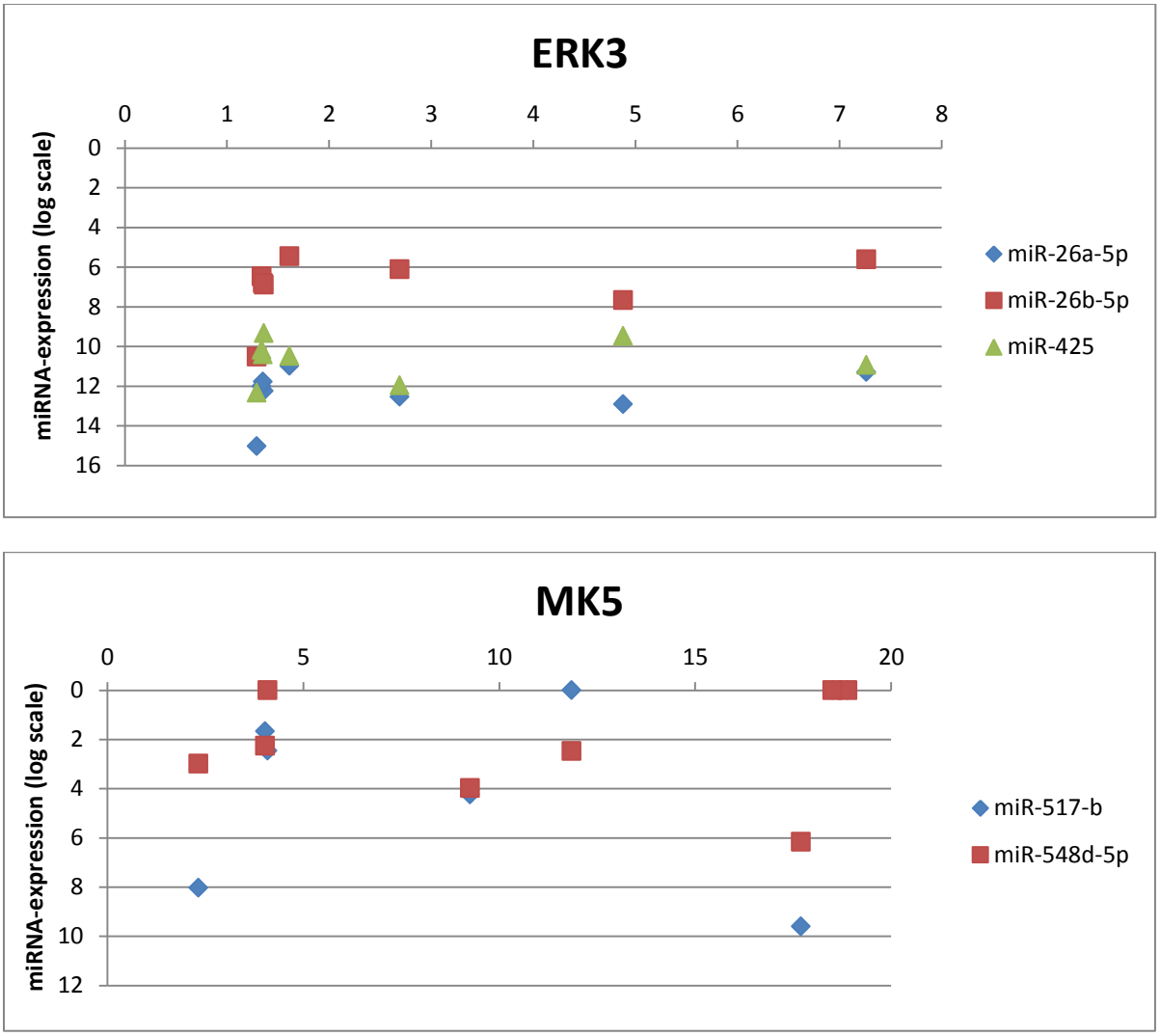

Figure 17. The correlation between the expression of ERK3 or MK5 in the different BCCL, and the miRNAs highly expressed or expressed in the same BCCL (see Error! Reference source not found. **and** Error! Reference source not found.**).** Values on the primary vertical axis are represented as an inverse scale showing the expression of ERK3/MK5. The horizontal axis is showing the expression of miRNAs (log values).

For ERK3, we can see that in the majority of the BCCL, there is an inverse correlation between the miRNA and the ERK3 expression (see Table 8). This may suggest a possible miRNA-mediated regulation of the ERK3 protein level in these BCCL. However a verification of a potential miRNA mediated ERK3 regulation remains to be identified or confirmed experimentally.

In the case of MK5, some of the miRNAs had medium to high expression in a few cell lines, while in others there was a low, or no expression of our selected miRNAs (see Table 9). No apparent correlation between the expression of MK5 and the miRNAs miR-517-b and miR-548d-5p (the miRNAs with the highest expressions of our selected in the BCCL (see Figure 16) was observed. Still, as shown in Table 9, the BCCL with the highest expression of MK5 (DU4475, MA11 and MCF7) display a very low or no expression of the miRNAs with predicted binding sites in the MK5 3'UTR. This observation may indicate a possible correlation between the MK5 expression and the expression of the selected miRNAs; at least a potential miRNA-mediated regulation of MK5 in BCCL is not excluded. However such an interaction would have to be identified and verified experimentally. Our search for putative miRNAs was only based on target sequence complementarity and binding affinity to the 3'UTR sequence of our reporter plasmids. The tissue-specific nature of miRNA-function was not taken into account. This might be the reason why some of the miRNAs are showing no expression in the different BCCL, although it does not rule out the possibility of MK5 regulation by these miRNAs.

BCCL	ERK3	miR-26a-5p	miR-26b-5p	miR-425	miR-302a-5p	miR-1297
AU565	1,29	15,02	10,51	12,33	0	0
DU4475	7,26	11,29	5,61	10,94	0	0
HCC1187	1,61	10,98	5,46	10,48	0	0
HCC1569	4,88	12,91	7,66	9,47	0	0
Hs578T	1,36	12,24	6,89	9,33	0	0
MA11	1,34	12,01	6,46	10,17	0	0
MCF7	1,35	11,78	6,81	10,41	0	0
MDAMB231	2,69	12,54	6,12	11,97	0	0

Table 8. The expression of ERK 3 and the pertaining miRNAs in different breast cancer cell lines (BCCL). miR-26a-5p, miR-26b-5p and miR-425 are highly expressed (also seen in Figure 16), compared to a relatively low expression of the ERK 3 protein in the same BCCL. miR-302a-5p and miR-1297 are not expressed in these breast cancer cell lines.

BCCL	MK5	miR-517b	miR-944	miR-1284	miR-1305	miR-548d-5p	miR-518d-5p	miR-559
AU565	17,7	9,59	0	0	0	6,16	0	0
DU4475	18,89	0	0	0	0	0	0	0
HCC1187	4,02	1,66	0	0	0	2,26	0	0
HCC1569	9,26	4,24	0	0	0	3,98	0	0
Hs578T	2,23	8,03	1,91	0	2,22	2,98	0	0
MA11	18,5	0	0	0	0	0	0	0
MCF7	11,85	0	0	0	0	2,47	0	0
MDAMB231	4,04	2,46	1,79	0	0	0	0	0

Table 9. The expression of MK5 and the pertaining miRNAs in different breast cancer cell lines (BCCL). miR-517-b and miR-548d-5p showed a higher expression in several of the breast cancer cell lines than the other miRNAs (also seen in Figure 16). The cell lines showing the highest expression of MK5 (DU4475, MA11 and MCF7), showed a low or no expression of the selected miRNAs with predicted binding sites in the MK5 3'UTR.

4 Discussion

Today, most of the physiologic miRNA-targets still remain to be verified experimentally (221). Although perfect seed pairing may not be necessary for miRNA – target 3'UTR interaction (222), the approaches that are being used for predicting target genes have a focus on identifying the evolutionarily conserved sequence, and comparing complementary elements in the targets 3'UTR to the miRNA seed sequence (223). This generally causes a high false positive- and false negative rate, resulting in high percentage of non-functional predicted target sites (221, 224).

miRNAs are important regulators of cellular mechanisms, also associated with cancer development, such as cell growth, differentiation and apoptosis . The role of each miRNA is dependent upon the biological role of their target. The growing discoveries in research and identifications of disease-specific miRNAs may lead to new prognostic tools and therapies, as well as individualized therapy which would be a huge advantage in such heterogeneous diseases as breast cancer. Many miRNA genes have been identified and proven, but there are still very few corresponding targets that have been identified and experimentally validated (221).

Cellular mechanisms are mediated and regulated in part through extracellular signals being conveyed to components inside the cell and cell nucleus. The ERK3/4-MK5 pathway has been implicated in processes involved in cancer development and progress, but in general there is not much knowledge surrounding these atypical protein kinases, as to how they are regulated or what their targets are (209). Extracellular signals for cell growth, survival, proliferation and differentiation are relayed through signaling pathways such as the MAPK/Erk pathway to intracellular responses, also mediated by the MAPK-activated protein kinases (MKs), such as MK5. The MKs are stimulated by mitogenic,- and stress stimuli, and are activated through phosphorylation of their kinase domain by extracellular signal-regulated kinases 1/ 2 and p38 MAPKs (225). As mentioned earlier, MK5 is also a known substrate of the atypical kinases ERK3/4 (63).

Initially, we wanted to assess the presence of the atypical MAPKs in different breast cancer cell lines, so we performed a western blot analysis on 8 different BCCL belonging to 3 different molecular subtypes (see Table 1). As shown in the results (see Figure 8 and Figure

9) we chose to compare the direct integrated intensities between the cell lines in the individual blots of ERK3 and MK5. This decision was based on the fact that prior to the western blot analysis, we had performed a protein assay to ensure equal loading of the samples, and also because the results from normalization with anti-actin antibody showed a larger variation of actin-expression in the different breast cancer cell lines than expected. This is a known phenomenon, where in disease states and during growth and differentiation, the expression of beta-actin can change in response to biochemical stimuli (213).

Our results showed expression of both ERK3 and MK5 in all of the 8 BCCL, although in varying levels. We could not see any correlation between the expression of the kinases and the molecular subtypes of the cell lines. For example, the expression of ERK3 varied significantly in the basal-A subtype (DU4475, HCC1187 and HCC1569, marked in green in Figure 8), which is associated with cancers that are invasive, aggressive, high grade and have poor prognosis and often triple-negative phenotypes (16-18).

The expression of ERK3 in the other two molecular subtypes of BCCL were much lower, and did not vary as much as in the basal-A subgroup. The luminal-subgoup (BCCL AU565 and MCF7) is associated with breast tumors that have good prognosis compared to the basal-like and ERBB2-positive tumors, although luminal-B differs from luminal-A in that it is HER2-positive, has a higher proliferative rate and histological grade, giving it a worse prognosis (8, 13, 14). The basal-B subgroup (Hs578T, MA11 and MDAMB231) also had a lower expression of ERK3 than the basal-A subtype of BCCL, with the exception on a slightly higher expression in MDAMB231. This subgroup of cell lines is associated with basal-like and ERBB2-positive tumors. They are aggressive, invasive, usually higher grade and have a higher risk of earlier relapse (12, 16-18).

When comparing the expression levels of the MK5 protein in the different BCCL, no significant correlation between the molecular subtypes of was observed. HCC1187 (basal-A), Hs578T (basal-B) and MDAMB231 (basal-B) had the lowest expression of MK5. AU565 and MCF7 (luminal) had a slightly higher overall expression of MK5, however DU4475 (basal-A) and MA11 (basal-B) displayed the highest MK5 expression levels.

Overall, these results demonstrate a general presence of the atypical kinases ERK3 and MK5 in BCCL of different molecular subtype. Nevertheless, it is difficult to draw any conclusions or trends from these results since variations between expressions in each subgropup were so

profound, and knowledge suroounding the topic of the atypical kinases and breast cancer is very limited.

Having established the consistent expression of ERK3 and MK5, we wanted to explore a potential regulation of the atypical kinase pathway by miRNAs. Indeed miRNAs are involved in the regulation of the regular mitogen-activated protein (MAP) kinase pathways both at transcriptional or translational levels, and often by targeting their 3'-untranslated regions (11, 92). Except for miR-34b/c, no other miRNAs have been linked to the regulation of ERK3/4-MK5 atypical MAP kinase signalling pathway (211).

Through TargetScanHuman, microRNA.org and DIANA LAB bioinformatics approaches, we selected 16 different miRNAs with potential binding target sites in the 3'UTR of the mRNAs of ERK3, ERK4 and MK5 (see Table 7). Since miRNA target silencing can occur both at the level of translation and mRNA degradation, we first aimed to investigate if the expression of our selected miRNAs would affect the mRNA levels of ERK3, ERK4 or MK5 in HEK-293T cells by using real-time RT-(q)PCR.

Our results showed no significant decrease in the cellular mRNA level of either ERK3, ERK4 or MK5 upon expression of any of the miRNAs with in silico predicted target sites in the 3'UTR of each of these kinases (see Figure 10**,** Figure 11 and Figure 12). However, this did not rule out the possibility of regulation through translational repression (95). Notably, the selection of our putative miRNAs was based on the potential binding-interactions to the truncated 3'UTR-sequence in the Mission LentiGoClone constructs. The fact that the available sequence was missing parts of the 3'UTR for ERK3/4 and MK5 mRNAs, limited our options in the search for putative miRNAs which could target this particular sequence. This leaves the possibility of a potential transcriptional regulation of ERK3/4 and MK5 by other miRNAs with possible target binding sites in other parts of the ERK3/4 and MK5 3'UTR or -mRNAs.

Since we saw no change in mRNA levels of ERK3, ERK4 or MK5 in any of the HEK-293T cells transfected with different miRNAs in the real time qPCR experiment (see Figure 10, Figure 11 and Figure 12), we made an attempt to identify a possible regulation on the translational level. The most commonly used approach to verify a miRNA target site is by using a reporter gene assay where the 3′ UTR of a predicted mRNA target is linked onto a luciferase reporter. A change in luciferase expression will indicate whether a miRNA binds to the 3' UTR and regulate the expression of the gene at the mRNA or protein level (221).

To determine if any of our selected miRNAs regulated ERK3, ERK4 or MK5 at the level of translation, the stably transfected cell lines containing the ERK3-, ERK4- or MK5 3'UTR fused to RenSP were reverse transfected with the pertaining miRNAs. Due to limited time and an elaborate process of ascertaining stably transfected cells containing the 3'UTR - renilla luciferase constructs, we were only able to carry out one biological replicate.

As seen in Figure 13, we saw no significant change in the relative luciferase activity in the transfected constructs compared to the controls for any of the miRNAs transfected in the HEK293Tcells expressing ERK4 3'UTR- RenSP. The luciferase-activity was measured 48 hours after transfection, replicating the conditions tested in the flow cytometry experiment (see method 2.13) to achieve comparable transfection efficiency. Results from the luciferase assay (see method 2.17) clearly indicated that none of the chosen miRNAs had a binding interaction with the part of the ERK4 3'UTR tested. However, this does not rule out potential binding to other parts of ERK4 3'UTR or -mRNA, with subsequent regulation of the protein expression.

As stated in the results, section 3.4, in the experiment aiming to identify a possible translational miRNA regulation of ERK3, the results showed huge variations in the relative luciferase activity in the N.T control and a fairly low relative luciferase activity in the N.C miRNA control (see Figure 14). It is impossible to draw any firm conclusions from such results, especially when the variations affect the control-samples which all the other samples are related to. As a result of this, we could look at the outcome in two different ways: 1) the expression of miR-26a-5p, miR-425 and miR-1297 seems to cause an up-regulation of the luciferase expression, if related to the N.C. miRNA, or 2) the expression of miR-26b-5p and miR-302a-5p cause a down-regulation of the luciferase expression, if compared to the P.C. construct. The second option seems more likely because the relative luciferase activity of the cells transfected with miR-26a-5p, miR-425 and miR-1297 are comparable to that of the P.C. construct. This construct expresses the ERK3 3'UTR- RenSP, without a potential miRNA-binding site, so the luciferase activity should not be affected by miRNA-mediated regulation. Therefore the expression of ERK3 3'UTR – RenSP could be interpreted as not affected by miR-26a-5p, miR-425 and miR-1297. At the same time it looks as though there is a potential binding to the ERK3 3'UTR by miR-26b-5p and miR-302a-5p causing a subsequent down-regulation of the luciferase expression, suggesting a regulation at the translational level by these two miRNAs.

In the experiment aiming to identify a possible translational miRNA regulation of MK5, the control samples also showed substantial differences in the relative luciferase activity (see Figure 15). In this case the N.C. miRNA seems as the right control due to the fact that its luciferase activity is similar to that of the P.C. construct and to the cells transfected with miR-944 and miR-1284. Interpretation of these results suggests a potential binding of miR-517-b, and miR-548d-5p, miR-518-d-5p and miR-559 to the MK5 3'UTR, and a possible subsequent down-regulation of the MK5 protein expression.

Since we only had a small part of the 3'UTR-sequence in our vector plasmid constructs, this might have resulted in an inaccurate accessibility for the miRNAs. We might also have missed other miRNA-mRNA interactions which could have participated in regulation of the protein expressions of ERK3/4 and MK5 by binding to the omitted 3'UTR-sequence or other parts of the transcripts. It would therefore have been optimal to include the entire 3'UTR sequence of the target in the experiment (221). Reporter assays can also be misleading in that higher concentrations of the miRNA than the physiological concentrations are being transfected into the cells, and may lead to an interaction that normally would not happen in the body. One can note rule out that ectopic overexpression in a more or less random cell line can be far from the physiological situation in which miRNAs work. Also, the lack of a co-factor environment may as well lead to a non-physiological interaction. Therefore, it is important to further experimentally validate the putative miRNA binding sites based on their physical interaction *in vivo* (221).

In an attempt to verify the results from the luciferase reporter gene assays in a biologically relevant context, we wanted to explore the expression of the selected miRNAs predicted to bind in the 3'UTR of ERK3 and MK5 (see Table 7) in BCCLs. When comparing to the results from the luciferase reporter gene assays, no simple correlation is found. Of the two miRNAs that caused a probable down regulation of ERK3 3'UTR – RenSP expression, only miR-26b-5p is detected in the cell lines. Equally, of the four miRNAs that caused a probable down regulation of MK5 3'UTR – RenSP expression, only miR-517-b, and miR-548d-5p are detected in the cell lines and in either case no clear correlation between miRNA and protein expression is evident. However some interesting tendencies were discovered through this biologically relevant system (see Figure 16, Figure 17, Table 8 and Table 9).
As mentioned in the results section (3.5 and Figure 16), it seems like the miRNAs expressed in the different BCCL can be divided into three groups: 1. those that generally are expressed

at a high level in all the breast cancer cell lines (BCCL) (miR-26a, miR-26b and miR-425), 2. miRNAs expressed, but not highly expressed, in all or most BCCL (423-5p, miR-34c-5p, miR-517-b, and miR-548d-5p) and 3. miRNAs that show a very low or no expression in any of the cell lines (miR-922, miR-124-5p, miR-302a-5p, miR-1297, miR-944, miR-1284, miR-1305, miR-518d-5p, and miR-559). The diverse miRNA expression pattern can probably be explained by the fact that miRNAs are highly tissue specific with highly variable expression patterns in different tissues (220).

When comparing the expression of the ERK3-protein in the different BCCL to the pertaining miRNA-expressions in the same cell lines we observed an inverse correlation to the miRNAs expressed (see Table 8). The ERK3-expression is relatively low, while three miRNAs, miR-26a, miR-26b and miR-425, show a relatively high expression in all the BCCL. However, in the luciferase reporter assay (method 2.17), only miR-26b caused a potential down-regulation of the luciferase expression. These results may suggest a possible miRNA-mediated regulation of the ERK3 protein level by miR-26b in these BCCL. Still a verification of a potential miRNA mediated ERK3 regulation remains to be identified or confirmed experimentally. In the case of MK5, there was an overall low or no expression of the miRNAs with predicted binding sites in the MK5 3'UTR. Interestingly, in the BCCL with the highest expression of MK5 (DU4475, MA11 and MCF7) almost none of the selected miRNAs were detected. In fact, mutual exclusive expression of miRNAs and their targets has been shown on a large scale by independent studies in mammals (226), and flies (227). Although a mutual exclusive expression of miRNAs and MK5 may exist a possible interaction between these miRNAs and MK5 would have to be verified experimentally.

Studies have shown that the ERK3/4-MK5 pathway can participate in several processes that are deregulated in cancer, including cell proliferation and -motility, invasiveness, and angiogenesis. Nevertheless, even though ERK3 expression has been reported to be upregulated in breast cancer (228), no clear role for either of the members of the ERK3/4-MK5 pathway in breast cancer is currently evident. The fact that the physiological role of the ERK3/4/ and MK5 signaling module is still enigmatic, lead us to explore a potential miRNA mediated regulation of these kinases. Despite the fact that a substantial part of our results are hampered by a limited amount of time, few biological and technical replicates and non-conclusive results, we revealed a possible miRNA mediated regulation of ERK3 and MK5 expression. In cancer there is a general need for elucidation of the deregulated RNA and protein networks and pathways in which miRNAs participate or contribute. This includes

miRNA target identification and verification, miRNA/phenotype associations and generation of network models of miRNA function. Undoubtedly, the future will bring more details, which will shed light on the role of the atypical kinases ERK3, ERK4 and MK5 in cancer and contribute to solve the puzzle of what regulates their expression.

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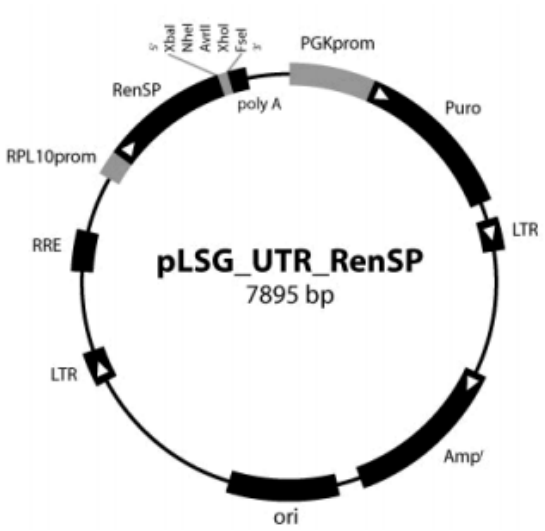
APPENDIX I

The MISSION® 3'UTR Lenti GoClone™ structure used in transfection experiments (from Sigma-Aldrich, SwithcGear Genomics Technical Bulletin):

Features of
Lentiviral Plasmid Vector pLSG_UTR_RenSP

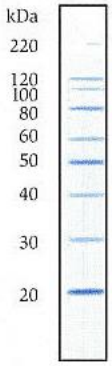


Name	Description
RPL10prom	Constitutive human RPL10 promoter
RenSP	Optimized *Renilla* luciferase gene
XbaI, NheI, AvrII, XhoI, FseI	Multiple Cloning Site for human 3'UTR insertion
PGKprom	Human phosphoglycerate kinase eukaryotic promoter
Puro	Puromycin resistance gene for mammalian selection
LTRs	Long terminal repeats
Amp^r	Ampicillin resistance gene for bacterial selection
ori	Origin of replication
RRE	Rev response element

APPENDIX II

MagicMark™XP Western Protein standard (from Invitrogen):

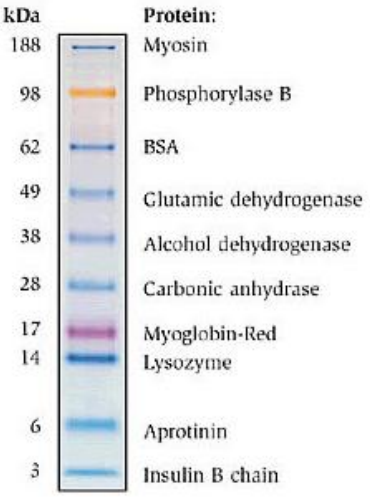
MagicMark™ XP Standard visualized with Coomassie® stain on an SDS-PAGE gel



NuPAGE® 4-12% Bis-Tris Gel with MES SDS Running Buffer, stained with SimplyBlue™ SafeStain.

SeeBlue®Plus Pre-Stained Standard 1X (from Invitrogen):

Apparent molecular weights of SeeBlue® Plus2 Pre-Stained Standard on a NuPAGE® Novex 4-12% Bis-Tris Gel w/MES



APPENDIX III

Potential microRNA-binding sites in the 3'UTR-region of the genes MAPK4 (ERK4), MAPK6 (ERK3) and MAPKAPK5 (MK5).

The following 3'UTR sequences are cloned with luciferase in the constructs from Sigma, called Mission® 3'UTR Lenti GoClone™:

MAPK6- 3'-UTR:

TGCTATGAAATCTTCCCCTCAAATTCCTCATCAAACATACAGCAGCATTCTGAAA
CATCTGAACTAAAACACTCAGCAGACATTTATCTTTGTATTCTTCATGAAATGTGT
TTTGTCTTTTTTTATTACTAGTGTTTAAGTCATTTTTTACTTGAATCAGATGGTGTC
ATTTAGTAAGGATTTTATGAGTTCTTGTTTTTTAAAATCCAGACTTTCTTTTTCTAC
ATGTGAGATAGTTTTCATTTTAACTGGCAT

MAPK4- 3'-UTR:

ACAATAAACTGGGCGACCTCAATGGTGCGTGCATCCCCGAGCACCCTGGCGACCT
CGTGCAGACCGAGGCCTTCTCCAAAGAAAGGTGGTGAGGGCGGAGGGGCCGCTC
CAGGCCCCACAGAGCAGGAGACCCCCAGAGAAAGCCGGGGCTGGCAGGAGGCG
GCCGCCCTTCCCGCCCCTCTCTGCTGCCTTGGGGTTGGCAGAACACGTGAAGGAT
CCGAGGAGCGAGAGGAATGTCCATTTCTTAAACTGCCT

MAPKAPK5-3'-UTR:

AGGTGATAGAAGAGCAAACCACGTCCCACGAATCCCAATAATGACAGCTTCAGA
CTTTGTTTTTTTAACAATTTGAAAAATTATTCTTTAATGTATAAAGTAATTTTATGT
AAATTAATAAATCATAATTTCATTTCCACATTGATTAAAGCTGCTGTATAGATTTA
GGGTGCAGGACTTAATAATAGTATAGTTATTGTTTGTTTTTAAGAAAAGCTCAGTT
CTAGAGACATACTATTACTTTAGGACTGTGTA

APPENDIX IV

Table 10. Putative microRNAs for potential binding of the 3'UTR-sequences in the 3'UTR Lenti GoClone of the genes *MAPK4* (ERK4), *MAPK6* (ERK3) and *MAPKAPK5* (MK5).

MAPK4	MAPK6	MAPKAPK5
hsa-miR-423-5p (7 mer) 3'UTR position: 61-89	hsa-miR-26a (9mer) 3'UTR position: 60-88	hsa-miR-517-b (7mer) 3'UTR position: 160-188
hsa-miR-922 (9 mer) 3'UTR position: 68-96	hsa-miR-26b (9mer) 3'UTR position: 60-88	hsa-miR-944 (7mer) 3'UTR position: 61-89
hsa-miR-34c-5p (6 mer) 3' UTR position: 70-98	hsa-miR-302a-5p (8mer) 3'UTR position: 45-73	hsa-miR-1284 (9mer) 3'UTR position: 93-121
hsa-miR-124 (6 mer) 3'UTR position: 72-100	hsa-miR-425 (8mer) 3'UTR position: 75-103	hsa-miR-1284 (6mer) 3'UTR position: 124-152
	hsa-miR-1297 (9mer) 3'UTR position: 60-88	hsa-miR-1305 (7mer) 3'UTR position: 9-37
		hsa-miR-548d-5p (8mer) 3'UTR position: 175-203
		hsa-miR-518-d (7mer) 3'UTR position: 159-187
		hsa-miR-559 (8mer) 3'UTR position: 175-203

APPENDIX V

Table 11. Through FLOW Cytometry analysis, reverse transfection showed higher transfection efficiency than traditional transfection:

Transfection method	Sample	Percentage (%) cells not-fluorescent	Percentage (%) of cells fluorescent
Traditional transfection	HEK-293T	100	0
		100	0
		100	0,036
	Control (LF)	100	0
		100	0,015
		100	0,03
	N.C. miRNA mimic	100	0
		100	0,016
		100	0,047
	N.C. FAM mimic	35,6	64,4
		33,2	66,8
		31,5	68,5
	N.C. FAM mimic	35,9	64,1
		32,3	67,7
		31,5	68,5
	N.C. FAM mimic	32,3	67,7
		30,4	69,6
		30,9	69,1
	N.C FAM mimic	36,6	63,4
		34,5	65,5
		31,9	68,1
	GFP	11,3	88,7
		10,7	89,3
		9,99	90
		11	89
	GFP	29,6	70,4
		29,7	70,3
		29,8	70,2
Reverse transfection	N.C miRNA mimic	99,9	0,088
		99,4	0,578
		99,5	0,452
		99,3	0,714
	Control (LF)	99,9	0,109
		99,9	0,128
		99,9	0,125
	N.C FAM mimic	0,944	99,1
		0,94	99,1
		0,629	99,4
	GFP	2,14	97,9

APPENDIX VI

Results from the protein assay:

Unknowns

Sample	Wells	OD_Values	Concentration	MeanConc	SD	CV	Dilution	AdjConc
Un01	C1	0,838	5,888	5,799	0,126	2,2	1,0	5,799
	D1	0,826	5,710					
Un02	C2	0,277	0,830	0,850	0,028	3,3	1,0	0,850
	D2	0,283	0,870					
Un03	C3	0,430	1,841	1,845	0,005	0,3	1,0	1,845
	D3	0,431	1,849					
Un04	C4	0,119	-0,056	-0,042	0,019	45,5	1,0	-0,042
	D4	0,124	-0,029					
Un05	C5	0,371	1,429	1,516	0,123	8,1	1,0	1,516
	D5	0,397	1,603					
Un06	E1	0,313	1,053	1,049	0,005	0,5	1,0	1,049
	F1	0,312	1,045					
Un07	E2	0,357	1,338	1,383	0,063	4,6	1,0	1,383
	F2	0,371	1,427					
Un08	E3	0,197	0,367	0,364	0,004	1,1	1,0	0,364
	F3	0,196	0,361					
Un09	E4	0,310	1,036	1,072	0,050	4,7	1,0	1,072
	F4	0,322	1,108					
Un10	E5	0,319	1,093	1,154	0,087	7,6	1,0	1,154
	F5	0,339	1,216					

Standards (mg/ml)

Sample	Concentration	BackCalcConc	Wells	OD_Values	MeanODValue	SD	CV
St01	10,000	15,838	A1	1,177	1,090	0,122	11,2
		8,898	B1	1,004			
St02	5,000	4,984	A2	0,770	0,640	0,184	28,8
		2,438	B2	0,509			
St03	2,500	3,125	A3	0,591	0,556	0,050	9,0
		2,528	B3	0,521			
St04	1,250	1,890	A4	0,437	0,463	0,037	7,9
		2,277	B4	0,489			
St05	0,625	0,899	A5	0,288	0,302	0,019	6,2
		1,064	B5	0,315			
St06	0,313	0,332	A6	0,191	0,180	0,015	8,6
		0,212	B6	0,169			
St07	0,156	-0,075	A7	0,116	0,106	0,013	12,6
		-0,172	B7	0,097			
St08	0,078	-0,284	A8	0,075	0,076	0,002	2,3
		-0,271	B8	0,077			

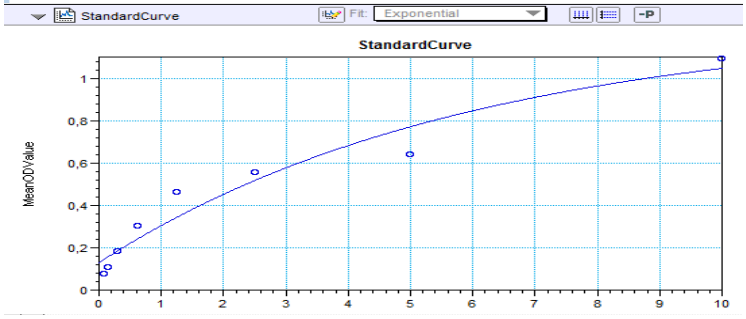


Figure 18. Results from the protein assay. Adjuvant concentrations (AdjConc) are given in µg/µl. Un01: DU4475, Un02: DU4475 sample 2, Un03: HCC1569, Un04: HCC1569 sample 2, Un05: HCC1187, Un06: Hs578T, Un07: AU565, Un08: MDAMB231, Un09: MCF7, Un10: MA11. Samples used for western blot were Un02, Un03, Un05 and Un06-10. Eight protein standards were prepared with albumin and MKK-lysis buffer.

Unknowns

Sample	Wells	OD_Values	Concentration	MeanConc	SD	CV	Dilution	AdjConc
Un01	C1	0,171	0,858	0,880	0,031	3,5	1,0	0,880
	D1	0,177	0,902					
Un02	C2	0,187	0,971	0,965	0,008	0,8	1,0	0,965
	D2	0,185	0,960					

Standards (mg/ml)

Sample	Concentration	BackCalcConc	Wells	OD_Values	MeanODValue	SD	CV
St01	10,000	Range?	A1	0,825	0,730	0,134	18,4
		6,721	B1	0,635			
St02	5,000	3,792	A2	0,479	0,532	0,075	14,1
		5,547	B2	0,585			
St03	2,500	2,360	A3	0,354	0,377	0,032	8,4
		2,824	B3	0,399			
St04	1,250	1,256	A4	0,226	0,235	0,013	5,7
		1,402	B4	0,245			
St05	0,625	0,713	A5	0,149	0,152	0,003	2,3
		0,746	B5	0,154			
St06	0,313	0,347	A6	0,092	0,090	0,003	2,9
		0,324	B6	0,088			
St07	0,156	0,125	A7	0,055	0,056	0,002	3,5
		0,141	B7	0,058			
St08	0,078	-0,022	A8	0,029	0,027	0,002	8,5
		-0,041	B8	0,026			

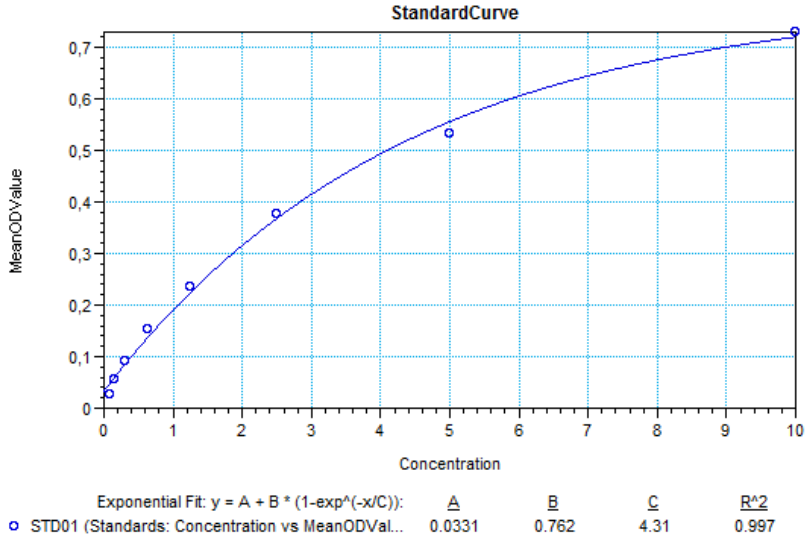


Figure 19. Results from protein assay of MDAMB231 (Un01 and Un02), prepared in the same way as in figure 1, used in the second MK5 blot.

APPENDIX VII

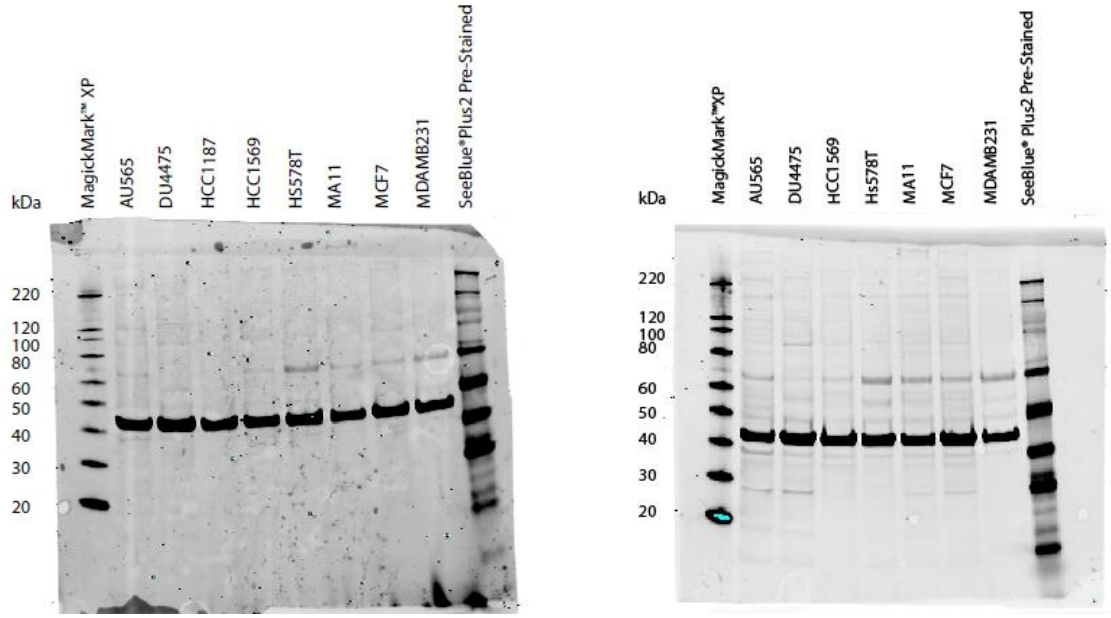


Figure 20. Expression of actin in breast cancer cell lines. Actin-control on the blots of MK5 (see figure 1). Anti-actin (1:1000) was used as the primary antibody. 680 CW anti-rabbit (0,8 µl) was used as the secondary antibody.

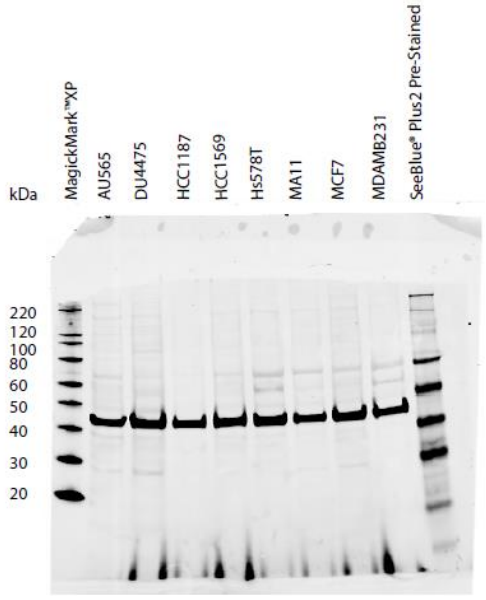


Figure 21. Expression of actin in breast cancer cell lines. Actin-control on the blot of ERK3 (see figure 3). Anti-actin (1:1000) was used as the primary antibody. 680 CW anti-rabbit (0,8 µl) was used as the secondary antibody.

Quantification of western blots shows variations in the expressions of the proteins MK5 and ERK3 between the different breast cancer cell lines.

Table 12. Results from the western blot quantification of β-actin on MK5 (see Figure 20, left-hand blot):

Sample	Actin control (I.I.)
AU565	115,62
DU4475	192,04
HCC1187	117,99
HCC1569	127,54
Hs578T	188,92
MA11	86,32
MCF7	192,76
MDAMB231	123,70

Table 13. Results from of the western blot quantification of β-actin on MK5 (see Figure 20, right-hand blot):

Sample	Actin control (I.I.)
AU565	190,78
DU4475	284,38
HCC1569	181,09
Hs578T	259,28
MA11	172,75
MCF7	326,52
MDAMB231	151,00

Table 14. Results from the western blot quantification of β-actin on ERK 3 (see Figure 21):

Sample	Actin control (I.I.)
AU565	56,71
DU4475	100,7
HCC1187	43,33
HCC1569	61,97
Hs578T	87,11
MA11	44,43
MCF7	89,76
MDAMB231	51,74